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Investigation of Titanium Dioxide Engineered Nanomaterials in Microbially Driven Environments

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Investigation of Titanium Dioxide Engineered Nanomaterials in Microbially Driven Environments

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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in

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by

Travis Waller

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ABSTRACT OF THE DISSERTATION

Influence of Engineered Nanomaterials on Colonic Health and Wastewater Treatment: An Assessment of Food and Industrial Grade Titanium Dioxides

by

Travis Waller

Doctor of Philosophy, Graduate Program in
Chemical and Environmental Engineering
University of California, Riverside, June 2018
Dr. Sharon L. Walker, Chairperson

This dissertation characterized two forms of TiO_2 engineered nanomaterials (ENMs) to determine the role of solution conditions in mediating differences in the environmental impacts of emerging nanomaterials. In general, ENMs designed for specific product matrices are functionalized to impart distinct characteristics capable of altering the environmental fate and transport (i.e. shape and surface composition). Observations of TiO_2 ENM release during product life cycles presents a concern as nanoparticles with unique characteristics begin accumulating in environmental systems. TiO_2 nanomaterials were introduced to three consecutive, environmental systems with microbial presence to characterize environmental implications of ENM presence, namely: a model human colon, septic tank, and a quartz sand filtration column.

The following critical findings resulted from this dissertation research. Colonic exposure to food (FG) and industrial (IG) grade TiO_2 inhibited a natural shift in microbial composition from *Proteobacteria* to *Firmicutes* phyla, with FG TiO_2 having the greater impact. Colonic pH levels decreased from IG and FG TiO_2 exposure where FG TiO_2 (pH 4) again had the greatest impact compared to IG TiO_2 (pH 5) and the control (pH 6). Therefore, inherent physical and chemical properties of FG and IG TiO_2 can produce different microbial responses in the colonic environment. Next, exposure of the septic system to IG TiO_2 particles had minimal effect on the composition of the denitrifying-dominant microbial community, while FG exposures resulted in a mixed functionality community less effective at waste treatment. Nano-FG TiO_2 exposure in septic systems may facilitate considerable changes in microbial community activity.

Lastly, FG TiO_2 exhibited a high degree of stability in septic conditions and both monovalent electrolyte suspensions. Elution of FG and IG TiO_2 was greatest in septic effluent at the higher nanoparticle concentration (100 ppm); however, FG TiO_2 was well retained at the low (2 ppm) concentration suggesting low elution from the drainage field.

Valuable insight into the role that inherent physical and chemical properties of emerging ENMs play in environmental systems is gained from this dissertation research. Additionally, the significance of the solution environment at mediating differences observed between uniquely, engineered nanomaterials sheds light on potential mechanisms for minimizing detrimental environmental implications of nanomaterials.

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Chapter 1

Introduction

Chapter 1: Introduction

1.1 Background and Motivation

Engineered nanomaterials (ENMs) are a broad and expanding class of manmade particles finding many applications due to their small size (< 100 nm on one side), large surface to volume ratio, and unique functional characteristics (Bishoge *et al.* 2018). ENMs represent a class of materials destined to expand its beneficial applications in medicine (Kreuter 1996; Martin *et al.* 2003), agriculture (López-Moreno *et al.* 2010), electronics (Song *et al.* 2012), water treatment (Thiruvenkatachari *et al.* 2008), food, pharmaceuticals, and personal care products (Weir *et al.* 2012; Keller *et al.* 2013). Medical applications of ENMs, for example, are often for targeted delivery of drugs and transporting medications pass the blood brain barrier (Kreuter 1996). Food and consumer products incorporate titanium dioxide (TiO_2) as a white pigment for optical aesthetics or as a matrix stabilizing ingredient (Weir *et al.* 2012). Evidence provided by these studies demonstrates the benefits of reduced size, and surface area to volume ratio, as well as, added functionalities. ENM design and manufacturing is a rapidly expanding industry developing smaller functionalized particles to improve the previously mentioned applications and uses.

Creation of nanosized particles inherently means the addition of *product or process* specific characteristics (Chan 2006) with potentially unknown behaviors in different environmental matrices (Dusinska *et al.* 2011). ENMs have been the focus of multiple environmental exposure and impact investigations considering consequences of nanomaterial usage (Li *et al.* 2007; Benn *et al.* 2008; Hamilton *et al.* 2009; Kobayashi *et* *al.* 2009; Moon *et al.* 2010; Gustafsson *et al.* 2011; Pablos *et al.* 2013; Mudunkotuwa *et al.* 2015). Numerous studies reported the release of ENMs from products at various points in product life cycles (Benn *et al.* 2008; Gottschalk *et al.* 2009; Kaegi *et al.* 2011; Keller *et al.* 2013; Fan *et al.* 2018; Giese *et al.* 2018) supporting the investigation of the environmental impacts of ENMs. ENMs found in certain products were found to leach by various mechanism from the intended application into numerous environmental settings including soil, air, and notably, both surface and ground water systems. Notably, microorganisms of ENMs at “point sources”, which includes WWTPs, are estimated to be at greater risk due to higher concentrations of nanoparticles (Giese *et al.* 2018). Nanoparticles have been shown to collect in the sediments of surface waters (Pablos *et al.* 2013) and TiO_2 ENMs, for example, in sediments facilitated increased absorption and uptake of heavy metals (Fan *et al.* 2018).

Multiple studies have considered the environmental behavior of engineered nanomaterials under idealized systems to elucidate mechanisms governing their fate and transport (Chen *et al.* 2007; Chen *et al.* 2007; Chowdhury *et al.* 2011; Huynh *et al.* 2011; Lanphere *et al.* 2013; Yang *et al.* 2014). The engineered nature of these particles indicates product or process specific manufacture or design enhancements (Chan 2006; Dusinska *et al.* 2011). As a result, early success in ENM usage from these enhancements encourages the development of increasing quantities of new, unique particles as more applications are discovered for them. Expansion occurring in this nature raises environmental impact concerns of keeping pace with potentially numerous combinations of unique particles and environmental matrices.

Characterization of the environmental fate and transport of ENMs in relevant environments, forms, and concentrations has been carried out previously to better detect nanomaterials and describe their environmental significance. Key observations included the detection and release of nanomaterials from food matrices (Weir *et al.* 2012), the estimation based on global material flows that wastewater treatment plants (WWTP) are an environmental system reached by numerous ENM applications with TiO_2 among the most abundant (Keller *et al.* 2013), and the detection and release of TiO_2 nanoparticles in real world, full scale WWTPs (Kiser *et al.* 2009; Westerhoff *et al.* 2011; Shi *et al.* 2016). Additionally, TiO_2 nanoparticle morphology was shown to affect the nanoparticle environmental behavior and implications by influencing the alignment of particles along the microbial surface altering particle toxicity in the idealized system as a result (Tong *et al.* 2013). Further, food grade (FG) TiO_2 also exhibited different characteristics in simple monovalent electrolyte conditions from more commonly studied forms of TiO_2 nanomaterials in parameters that affect its environmental fate and transport, such as particle mobility and elemental composition (Yang *et al.* 2014).

Evidence provided addresses that ENMs possessing unique characteristics can be present in and released from product matrices into environmental systems during the product lifecycle (Benn *et al.* 2008; Gottschalk *et al.* 2009; Kaegi *et al.* 2011). Further, ENMs such as TiO_2 are an environmental concern as they have extensive usage in foods and consumer products bringing them into direct contact with humans, microorganisms, and sensitive environmental systems including waterbodies and water treatment facilities (Weir *et al.* 2012; Keller *et al.* 2013). As such, the role of TiO_2 ENMs in microbially driven systems of the human body and water treatment are the focus of this study.

Frequent use of engineered TiO_2 nanoparticles in foods and consumer products such as cosmetics, food coatings, and pigments provides a direct pathway for nanomaterials to enter the human body (Weir *et al.* 2012; Keller *et al.* 2013). Inhibition and other deleterious effects have been shown to develop in model environmental organisms due to ENM ecotoxicity (Gurr *et al.* 2005; Hamilton *et al.* 2009; Tong *et al.* 2013). Ecotoxicity of TiO_2 nanoparticles to fish (Zhang *et al.* 2007) and rodents (Jani *et al.* 1994) observed intestinal accumulation resulted from exposure prior to the translocation of nanoparticles into other bodily systems. Koeneman *et al.,* (2010) also reported that TiO_2 nanomaterials could remain small and stable enough to pass through intestinal cell lining although particle aggregation would limit the quantity (Koeneman *et al.* 2010). Therefore, the presence of TiO_2 nanoparticles, the potential for deleterious effects on microorganisms, and the inhabitation of the human colon by a microbial community makes the large intestine a prime area of study for assessing environmental implications of TiO_2.

Microorganisms comprising the microbiome of the large intestine exist in an anaerobic environment and perform the role of further degrading digested food waste before excretion in bowel movements (Taylor *et al.* 2015). Symbiotic relationships develop between species to maintain or restore balance within the microbial community making it a highly adaptable and responsive environment (Arumugam *et al.* 2011). Bacteria produce extracellular polymeric substances (EPS) as part of normal metabolism, but also in response to changing environmental conditions (Van Loosdrecht *et al.* 1993; Gong *et al.* 2009; Flemming *et al.* 2010; Marcus *et al.* 2013). Quantifying biofilm growth (EPS) provides a phenotypic indicator of how the bacteria and system are changing in response to one another. Excessive biofilm formation, in addition to decreasing pH, within the human colon is thought responsible for numerous chronic inflammatory, ailments including Crohn's disease and ulcerative colitis (Raimundo *et al.* 1992; Fallingborg *et al.* 1993). As such, consideration of the microbial community within the colon provides insight into systemic effects resulting from TiO_2 nanomaterials in complex solutions to complement results observed in idealized studies. Additionally, waste from the human colon becomes the influent that reaches WWTPs which allows the examination of TiO_2 ENMs across consecutive systems.

Full scale, WWTP are designed to degrade organics and reduce nutrient loadings reintroduced to surface and groundwater systems to minimize anthropogenic contamination (Bitton 2005). WWTPs utilize two to four stages prior to effluent discharge: preliminary treatment to remove debris, primary treatment using physical separation, secondary treatment for biological or chemical nutrient removal, and tertiary treatment for additional removal of nutrients, pathogens, and toxins. Biological unit processes are the primary treatment method for wastewater treatment meaning microbial activity is vital to effective wastewater treatment (Bitton 2005). TiO_2 nanoparticle usage in foods and consumer products is a significant source contributing to its detection in surface and groundwater systems, as well as, wastewater treatment plants (Keller *et al.* 2013). Observations of the environmental release of TiO_2 nanoparticles (Weir *et al.* 2012), toxicity to aquatic species (Zhang *et al.* 2007; Hamilton *et al.* 2009), and bioaccumulation in both microorganisms (Zhang *et al.* 2007; Shi *et al.* 2016) and sediments (Praetorius *et al.* 2012) raise concerns regarding nano-TiO_2 in wastewater treatment systems, specifically with the impact to the microbial community driving these treatment processes. Nanoparticles studied to date tend to associate with biosolids and are removed during sedimentation (Kiser *et al.* 2009; Westerhoff *et al.* 2013); however, onsite waste treatment systems (i.e. septic system) are often simpler treatment processes without regular maintenance where solids accumulate over time (Canter 1985; EPA 2002).

Septic wastewater treatment systems are microbially driven, anaerobic digesters using only sedimentation and filtration treatment stages (Canter 1985). Microbial degradation of organics and sedimentation of suspended solids produces an effluent that is filtered through the soil drainage field prior to reaching the groundwater system. Septic systems, as of 2007, serviced an estimated 20% (~26 million) of homes in the United States (EPA 1999). Although the septic system is effective at treating household waste when properly designed, installed, and maintained, shortcomings in these areas result in septic system failure (EPA 1999). Waste stream loadings exceeding microbial activity or drainage field capacity ultimately lead to system breakdown (Canter 1985). Large quantities of nanomaterials detected at full scale WWTPs suggests similar ratios may be reaching the failure prone septic system, in addition to an extended time for microbial interactions due to biosolid association and accumulation (Taylor *et al.* 2015). TiO_2 and ENMs well studied and characterized in full scale WWTPs have largely been neglected in the onsite, septic systems including: impacts on system performance, effluent quality, TiO_2 transformation, and microbial community responses to presence of TiO_2 ENMs. Understanding TiO_2 ENM behavior in microbially driven wastewater systems is key to minimizing anthropogenic, environmental pollution. As such, it is critical that academic study and environmental regulations be based upon environmentally relevant forms and concentrations of nanomaterials.

Increasing production of uniquely behaving TiO_2 and other nanoparticles released from consumer products into environmental systems raises a key concern: whether the knowledge of the environmental implications of emerging ENMs can match pace with a rapidly expanding industry or if complex environmental systems and solutions can mediate particle differences. As such, the determination of similarities of inhibitory effects of TiO_2 ENMs in complex systems and solutions is vital for understanding the extent of their environmental influence and role.

1.2 Objective and Scope

The overall goal of this research was to elucidate whether observed differences in nanoparticle environmental behavior in idealized systems manifest in more complex systems and solutions. Specifically, this research focused on the environmental significance of food grade titanium dioxide (TiO_2) nanoparticles within microbially driven environmental systems and dissimilarities in environmental responses between different forms of TiO_2 nanomaterials. This work utilized a model human colon, septic wastewater treatment tank, and sand column to characterize (a) microbial communities in the human gut (Chapter 2) and (b) septic system microbial communities and function during and after exposure to TiO_2 nanoparticles (Chapter 3), as well as (c) filtration mechanisms governing removal of each nanoparticle post septic treatment (Chapter 4), respectively. TiO_2 nanoparticles represent a commonly detected nanomaterial at wastewater treatment facilities resulting from its frequent use in foods and consumer products. Multiple forms of TiO_2 were selected due to TiO_2 nanoparticles being regulated and studied as one unique, particle type yet industrial and food grade TiO_2 are known to possess functional differences. Phenotypic, genotypic, and biochemical parameters were used to monitor deviations in microbial community function.

The objectives of this work coincide with the larger goals of the university of California Center for Environmental Implications of Nanotechnology (UC CEIN). UC CEIN is a multidisciplinary research center focused on the study of responsible usage and safe implementation of nanotechnology.

1.3 Hypotheses and Specific Objectives

The hypotheses and specific objectives presented below were developed to address the overall goals of this research. Each of the three specific objectives is addressed in an independent chapter within this dissertation.

Hypothesis 1: Complex solution conditions of the colon medium will negate differences observed between food and industrial grade TiO_2 *nanoparticles in idealized environments resulting in negligible effect upon the microbial community of the human gut.*

Hypothesis 1 was tested by ascertaining differences in environmental responses of the human gut microbiome resulting from exposure to food and industrial grade TiO_2 nanoparticles. Differences in impact to microbial community structure and cellular function after exposure to food or industrial grade TiO_2 ENMs were specifically addressed in this investigation. This objective was accomplished utilizing an *in vitro* model human colon bioreactor inoculated once weekly with a microbial community donated by a healthy, 26 year old adult female. Food and industrial grade TiO_2 nanoparticles were introduced independently to the model colon bioreactor three times daily after an initial microbial inoculation. Nanoparticles were selected based on estimates of TiO_2 accounting for nearly half of ENMs detected in wastewater treatment facilities due to frequency of usage in foods, pharmaceuticals and personal care products (Keller *et al.* 2013). As such, while industrial grade TiO_2 is more commonly studied in literature, food grade TiO_2 represents a more environmentally relevant particle type (Weir *et al.* 2012). The purpose of this work was to determine the role of ENMs in the colonic environment and provides vital knowledge regarding potential deleterious impacts. Phenotypic, biochemical, and compositional responses were monitored across a five-day experimental week to observe deviations in (1) the microbial community structure determined via DNA sequencing, (2) the microbial community's ability to degrade substrates via biochemical analyses, and (3) changes in phenotypic expressions by physicochemical analyses when the system was exposed to ENM loadings. This work is significant to human health as the microbial community of the colon can facilitate the onset of chronic, intestinal ailments (Venema *et al.* 2003; Arumugam *et al.* 2011). (Chapter 2).

Hypothesis 2: Protein coronas and other coatings are expected to develop around each TiO_2 *nanoparticle and as a result, prevent direct interaction between the* TiO_2 *and microbial community of septic system, as well as, disrupt the waste degradation process by making substrates biologically unavailable.*

The goal of objective 2 was to test hypothesis 2 by determining the effect food and industrial grade TiO_2 nanoparticle presence had on the wastewater microbial community and on septic system waste treatment efficiency. TiO_2 ENMs released from product matrices during usage have been detected at full scale, wastewater treatment plants (Kiser *et al.* 2009; Westerhoff *et al.* 2011). Full scale, WWTPs typically rely on biological treatment and incorporate two to four treatment stages (pretreatment, primary, secondary, and tertiary) with each providing an additional level of effluent clarity (Bitton 2005). Notably, although the colloidal sizes aggregates were well removed, up to 70% of nano-TiO_2 had remained suspended in effluent and was reintroduced to surface waters (Kiser *et* *al.* 2009; Westerhoff *et al.* 2011). Septic system waste treatment, conversely, only utilizes biological treatment, sedimentation (primary treatment stage), and filtration (Canter 1985; EPA 2002). Septic systems are decentralized WWTP applications and therefore, would receive similar influents to full scale WWTPs which also means TiO_2 nanoparticle introduction. As such, the septic system represents an overlooked environment of concern regarding nanoparticle introduction to groundwater, and eventually surface water.

The purpose of this work was to characterize the microbial community and effluent quality of the septic system in response to exposure to food grade TiO_2 (environmentally relevant) and industrial grade TiO_2 (commonly studied). An *in vitro* model colon provided a reproducible microbial community and fecal matter for introduction to a bench scale, septic system. Food and industrial grade TiO_2 nanoparticles were introduced independently to the model colon bioreactor prior to introduction to the septic tank. Nanoparticles were selected based on prevalence of TiO_2 in wastewater treatment systems (Westerhoff *et al.* 2011). Microbial phenotype, community structure, and effluent quality served as indicators of system performance and perturbations from TiO_2 exposure and were monitored across 9 weeks. Microbial phenotype and composition provided indicators of biofilm growth and shifts in community structure (16S rRNA sequencing), respectively. Effluent quality quantified organic matter and system acidic buffering capacity. Findings of this study provide important information regarding ENM effect on microbially driven systems, as the bacteria in the septic system are the primary degraders of organic matter and system function is dependent on the microbial community (Crittenden *et al.* 2012). (Chapter 3).

Hypothesis 3: Food grade TiO_2 nanoparticles would remain stable after passing through the low ionic strength, near neutral pH septic system effluent allowing their elution into the groundwater system.

Objective 3 aimed to characterize the transport of food grade TiO_2 nanoparticles in porous medium to understand their subsurface behavior and removal potential. Detection of TiO_2 ENMs in receiving waters of WWTPs is indicative of particle elution from after the waste treatment process (Kiser *et al.* 2009; Westerhoff *et al.* 2011; Shi *et al.* 2016). While it has been reported that TiO_2 nanomaterials can be released back into the environment from WWTPs, much remains unknown about TiO_2 nanoparticle behavior after elution from the septic tank, specifically in the porous media drainage field. Low tendency for aggregation of food grade TiO_2 in high ionic strength solutions with proteins, carbohydrates, and multivalent salts revealed a high stability of this nanoparticle in suspension (Waller *et al.* 2017). The purpose of this work was to fill the knowledge gap of this vital environmental system (drain field) as it serves the role of minimizing anthropogenic pollution. This study characterized the transport behavior and mechanisms of food grade TiO_2 particles in porous media to reflect solutions conditions of septic effluent. Food and industrial grade TiO_2 were flowed at 2 and 100 ppm concentrations through a cylindrical, wet packed quartz sand column to assess removal potential and stability of nanomaterials after exposure to septic waste treatment. Characterization of nanoparticles included particle size and mobility within each solution condition. Normalized elution values (C/Co), hydrodynamic diameter, and zeta-potential were monitored to shed light on subsurface transport characteristics. Breakthrough curves were then created to elucidate the influence of solution chemistry and particle concentration in FG TiO_2 porous media transport. Findings of this study provide valuable information on the capacity for the septic system (tank and drain field) to minimize nanoparticle introduction to the septic system. (Chapter 4).

1.4 Experimental Approach

Food and industrial grade TiO_2 nanoparticles were selected as model nanomaterials due to their frequent use in pharmaceuticals, consumer, and personal care products. Food grade TiO_2 nanomaterials were donated by Dr. Paul Westerhoff of Arizona State University and originally purchased from a mainstream supplier based in China. Industrial grade TiO_2 nanomaterials were sourced from Evonik Degussa Corporation (Evonik Corporation, Parsippany, NJ, USA).

Three representative model environmental systems were selected to investigate the influence of food and industrial grade TiO_2 nanomaterials. A model, lab scale human colon bioreactor was designed and operated to mimic conditions of the large intestine/colon (Chapter 2). This system provided the reproducible microbial community utilized and monitored throughout the objectives of this dissertation. Excrement produced from the model colon reactor were then introduced into the second model system: the septic wastewater treatment tank (Chapter 3). The septic tank is a two-chambered anaerobic waste digester in which biological degradation is the primary function of organic waste removal from effluent. The final system utilized in this study is the cylindrical, quartz sand filled column operated to conduct nanoparticle filtration characterization experiments (Chapter 4).

This PhD dissertation comprises 5 chapters in total including the Introduction (Chapter 1) and the conclusion (Chapter 5). Following the introduction, Chapter 2 entitled “Food and Industrial Grade Titanium Dioxide Impacts to Gut Microbiota” characterizes phenotypic, biochemical, and compositional parameters of the colon bioreactor microbial community after exposure to food and industrial grade TiO_2. Both TiO_2 nanoparticle exposures interestingly resulted in a hindered shift of the microbial community composition with food grade TiO_2 causing the greater inhibition. Additionally, both TiO_2 exposures resulted in lower values of the colonic pH (< 5) as compared to the control (> 5), with food grade exposures recording the largest reduction (~ pH 4). Similar trends in microbial community hydrophobicity and electrophoretic mobility between baseline, food, and industrial grade exposures indicated TiO_2 exposures may have little effect on microbial stability. Generally, findings reflect that when considering environmental risk and exposure, as well as the design of environmental fate and toxicity studies that inherent physical and chemical properties of engineered forms of TiO_2 may indeed produce different microbial responses. The result of this study was published in 2017 in *Environmental Engineering Science* 34.8 (2017): 537-550.

Chapter 3 entitled “Influence of Food and Industrial Grade TiO_2 Nanoparticles on Microbial Diversity and Phenotypic Response in Model Septic System” considers phenotypic and compositional responses of anaerobic wastewater bacteria to food and industrial grade TiO_2 introduction to the septic treatment system. Effluent quality was additionally monitored to indicate any perturbations to septic system performance resulting from either TiO_2 nanomaterial. Distinct responses in select microbial and effluent quality parameters resulted from food and industrial grade TiO_2 exposure. Notably, a more diverse microbial community composition developed during food grade TiO_2 exposures, indicating that food grade TiO_2 may alter microbial relationships affecting anaerobic digestion efficiency. Results indicate that nano-food grade TiO_2 may cause considerable changes to microbial function in septic systems. Findings further support the notion that understanding downstream environmental impacts of nanomaterials requires studying environmentally specific and relevant forms of nanoparticles. This publication has been accepted to *Environmental Engineering Science.*

Chapter 4 entitled “Influence of Septic System Wastewater Treatment on Titanium Dioxide Nanoparticle Subsurface Transport Mechanisms” explored the three dimensional sand filtration behavior and removal mechanisms of food grade TiO_2 after exiting the septic system. FG and industrial grade TiO_2 (more commonly studied) were introduced to septic tank effluent and low ionic strength electrolyte solutions (4 mM KCl and 10 mM KCl) at 2 and 100 ppm concentration to characterize nanoparticle aggregation behavior and mobility in solution aggregation of the nanoparticles. Results reflect that FG TiO_2 aggregate size (200-400 nm) remained consistent across solutions and concentrations whereas IG TiO_2 was far more prone to aggregation. Column breakthrough curves indicate that elution of FG and IG TiO_2 was greatest in septic effluent at the higher nanoparticle concentration (100 ppm). FG TiO_2 (2 ppm) was well retained in septic effluent suggesting that particles remaining suspended in septic effluent may still be prevented from reaching the groundwater system, although eluted particles are expected to be highly stabilized. Findings provide valuable insight into the role of the solution environment at reducing differences between engineered nanomaterials in environmental systems. This manuscript is submitted to *Analytical and Bioanalytical Chemistry.*

Chapter 5, entitled "Summary and Conclusions" summarizes the findings and new understandings from this PhD research. Chapter 1 of this work is currently published while chapter 2 and 3 are under review. Manuscripts that have resulted from this work are listed below.

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Chapter 2

Food and Industrial Grade Titanium Dioxide Impacts to Gut Microbiota

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Chapter 2: Food and Industrial Grade Titanium Dioxide Impacts to Gut Microbiota

Abstract

Titanium dioxide (TiO_2), frequently used in foods, coatings, pigments, and paints, accounts for a major fraction of engineered nanomaterials released from products during usage. Impacts of food and industrial grade TiO_2 on the composition and phenotype of a human gut microbiota, representing an upstream anthropogenic system, were studied to elucidate systemic perturbations to the microbial community. Findings show an inhibition of an expected, natural shift in microbial composition observed during control conditions from Proteobacteria to Firmicutes phyla in the presence of both types of TiO_2 particles, with food grade exposures having a greater effect. Additionally, both TiO_2 exposures resulted in lower values of the colonic pH (< 5) as compared to the control (> 5), with food grade exposures recording the largest reduction (~ pH 4). Similar trends in microbial community hydrophobicity and electrophoretic mobility between baseline, food, and industrial grade exposures indicate TiO_2 exposures may have little effect on microbial stability. Results of this study indicate that inherent physical and chemical properties of the two TiO_2 forms may indeed produce different microbial responses, which is significant when considering environmental exposure and risk, as well as the design of environmental fate and toxicity studies.

2.1 Introduction

Engineered nanomaterials (ENMs) have applications within the industrial, consumer, medical, and numerous other industries resulting from their unique physical and chemical properties (Masciangioli et al. 2003; Klaine et al. 2008). These properties include enhanced electronic, optical, thermal, and photoactive capacities permitting their use in such a vast number of areas (Petosa et al. 2010). Consumer based applications for ENMs include foods, pharmaceutical, and personal care products for stabilization or optical properties (Keller et al. 2013). Medical ENM applications have improved drug delivery, allowed more efficient toxic substance detection and, enhanced medical diagnostics (Kreuter 1996; Long et al. 2001; Martin et al. 2003).

Assessing TiO_2 ENM toxicity to mammalian and aquatic organisms has been the subject of multiple studies. Ecotoxicological assessments of ENMs on fish (Zhang et al. 2007) and rodents (Jani et al. 1994) determined that TiO_2 exposures resulted in the intestinal accumulation of the metal oxide ENM before the subsequent translocation to other bodily systems, such as the liver and spleen. Further, an *in vivo* investigation of TiO_2 ENMs introduced to mice resulted in a secondary genotoxic response associated with DNA instability, inflammation, and/or oxidative stresses (Trouiller et al. 2009). TiO_2 ENMs introduced to an *in vitro* intestinal model possessed the capacity to cross the epithelial lining via transcytosis although these effects were nonlethal (Koeneman et al. 2010).

Particle morphology is an additional consideration of ENM ecotoxicty having been shown to cause inhibition and other deleterious effects to model environmental organisms (Gurr et al. 2005; Hamilton et al. 2009; Tong et al. 2013). Anatase TiO_2 exhibited greater toxicity than the rutile form in human bronchial epithelial cells resulting in DNA damage, lipid peroxidation, and micronuclei formation (Gurr et al. 2005). However, toxicity in this case could additionally result from differences in particle size as the anatase TiO_2 was 10-20 nm while the rutile form was 200 nm. An investigation between anatase TiO_2 nanospheres (60-200 nm diameter) and nanobelts (60- 300 nm diameter; 5 µm and 15 µm length) found that greater toxicity in alveolar macrophages and mice occurred in presence of the longer, nanobelts compared to the shorter, smaller spherical TiO_2 particles (Hamilton et al. 2009). Notably, nanobelt introduction resulted in inflammatory responses by alveolar macrophages and the release of inflammatory cytokines. More recently, the induced phototoxicity to model pathogens was attributed to morphological differences between spherical and low-dimensional TiO_2 ENMs (Tong et al. 2013). A major contributing factor was the exposed surface area based on the ENM alignment along the bacterial surface, mediating the capacity for production of the ROS species. Morphological studies reveal that the same nanomaterial (e.g. TiO_2) can result in different microbial impacts depending on the specific properties associated with the particle.

Average daily intake of TiO_2 ENMs in food and personal care products were quantified using a number of food sources (Weir *et al.* 2012). Adults in the US were found to potentially ingest up to 1 mg TiO_2/kg of bodyweight per day. Further, food grade forms of TiO_2 more commonly found in food and personal care products would predominate intake rather than industrial forms more commonly incorporated in environmental health and safety studies. Five different types of food grade TiO_2 in a separate study were obtained from global food suppliers and characterized for comparison to industrial grade (Aeroxide® P25, Evonik Degussa) highlighting notable differences between parameters linked to environmental fate and toxicity (Yang *et al.* 2014). Elemental and surface composition analyses revealed the presence of substances known to influence environmental fate and toxicity such as phosphorous, silica, and alumina in food grade samples, but non-detectable levels in industrial grade samples. These elements were stated to be the result of anionic surfactants added by the manufacturer to increase particle stability.

Considerable differences also existed between the food and industrial grade ENMs in the primary particle size and crystal structure (Yang et al., 2014). Food grade samples contained between 17% - 35% nanoparticles (<100 nm) TiO_2 compared to the industrial grade which had 100% of the particles in the nano-range. Four of the food grade samples had an exclusively anatase crystal structure whereas P25 and the remaining food grade sample were a combination of both anatase and rutile; however, the food grade sample was largely rutile while the industrial grade had greater anatase structure. Further, cationic dyes were observed to adsorb more readily to food grade than industrial grade particles, indicating different potentials for interaction with organics in the environment. These findings support a similar sentiment as Weir et al., (2012), that studies considering the fate and toxicity of ENMs should utilize food grade TiO_2 in addition to solely industrial grade as inherent differences exist between the particle types that are capable of altering their behavior in human and environmental systems.

This study aimed to assess whether the inherent differences between these unique TiO_2 particles (food vs industrial grade), such as particle size and surface potential resulted in comparable perturbations within a complex environment represented here by a human gut microbiota. Specifically addressed in this study were the differing impacts to the microbial community structure and cellular function in the presence of food or industrial grade TiO_2 ENMs. A lab scale, *in vitro* model human colon reactor was utilized to assess perturbances to (1) the microbial community structure determined via DNA sequencing, (2) the microbial community's ability to degrade substrates via biochemical analyses, and (3) changes in phenotypic expressions by physicochemical analyses when the system was exposed to ENM loadings.

2.2 Materials and Methods

2.2.1 Model Colon Reactor

The bench scale model colon reactor has previously been used in studies to determine impacts to microbial communities in the presence of a model pathogen (Marcus et al. 2013), as well as impacts of other ENMs less commonly used in food and personal care products on the community function (Taylor et al. 2015). Model colon dimensions and operation have been described previously (Marcus et al. 2013).

Briefly, the cylindrical glass reactor is housed in an incubator (Barnstead MaxQ 4000; Thermo Scientific, Asheville, NC) maintained at 37°C. Testing of each experimental condition — baseline (control), food and industrial grade TiO_2 exposures — spanned five days termed experimental "weeks" and each condition was performed in triplicate (e.g. three baseline weeks, three industrial grade exposure weeks, and three food grade exposure weeks). The colon medium (Venema et al. 2003), similar in composition to digested food entering the large intestine, is pumped into the reactor in 100mL volumes three times daily (simulating feedings) for the entire five days duration of the experiment while 100 mL are equally removed for testing. The three feeding times were the morning, afternoon, and night and remained constant between all 3 testing conditions. Included in the colon medium are sources of simple salts, carbohydrates, and proteins simulating a western diet. This medium replicates digested food entering the large intestine termed here as colon medium. Per liter the medium contains 4.5g NaCl, 2.5 g K 2HPO4, 0.45 g CaCl2·2H2O, 0.5 g MgSO4·7H2O, 0.005 g FeSO4·7H2O, 0.05 g ox bile, 0.01 g hemin, 0.4 g cysteine, 0.6 g pectin, 0.6 g xylan, 0.6 g arabinogalactan, 0.6 g amylopectin, 5 g starch, 2 ml Tween 80, 3 g Bacto peptone, and 3 g casein. After autoclaving the media, 1 ml of a vitamin mixture containing, per liter, 1 mg menadione, 2 mg D-biotin, 0.5 mg vitamin B12, 10 mg pantothenate, 5 mg nicotinamide, 5 mg para-aminobenzoic acid, and 4 mg thiamine (Minekus 1999).

The colon medium flowed (5 mL/min) within a dialysis tube inside the reactor while a polyethylene glycol mixture flowed external to the dialysis tube (2.1 mL/min), yet still within the reactor, simulating the dehydrating function of the large intestine. All microbial and colonic fluid characterization were performed in triplicate, twice daily at the morning and afternoon feedings. Before the start of the experimental week, colon medium (200 mL) was inoculated and incubated for 24 hours. The inoculum consisted of a microbial community extracted from human fecal material provided by a healthy, 26-year-old female who had been free of antibiotics for eight months. Initially, control experiments are conducted with the system operating in the absence of ENMs in the model colon. Subsequently, model ENMs are introduced, in independent experiments to evaluate perturbations to the microbial community under each exposure condition. The collected colonic fluid, 100 mL, is then utilized for microbial community characterization experiments. During weeks of TiO_2 addition the incubator and all media containers are covered to simulate dark conditions.

2.2.2 Nanoparticle Selection and Characterization

Two forms of nanoscale titanium dioxide were used individually during exposure experiments testing microbial community perturbations. An industrial grade TiO_2 (Aeroxide TiO_2 P25; Evonik Degussa Corporation, NJ) was selected as it is commonly used in environmental toxicity, fate, and transport studies (Chowdhury et al., 2013; Yang et al., 2014; Brunett et al., 2009; Maness et al., 1999). P25 has a nominal size of 21 nm and no surface coatings are stated in product specifications. A commercially available TiO_2, referred to as food grade in this study, was acquired from Arizona State University to represent a form more likely to be utilized in food and personal care products. Particle size was determined to be 122 ± 48 nm using transmission electron microscopy and ion chromatography revealed the surface was coated with inorganic phosphate (Yang et al., 2014).

The daily ENM dose introduced to the colon reactor was determined using estimates based on TiO_2 quantities extracted from commercial food products of approximately 0.3-0.7 mg/kg of body weight-day for an adult consuming a western diet (Weir et al. 2012). An assumed mass of 80 kg determined suggested approximately 36 mg of TiO_2 were consumed by an adult male each day (Walpole et al. 2012). ENMs were quickly added to the bulk solution of colon media at the start of the experimental week, after autoclaving the media, in the biological safety cabinet to minimize contamination risks.

Nanoparticle characterization allowed for better understanding the inherent properties and characteristics of the two different types of TiO_2. X-ray diffraction (XRD), dynamic light scattering (DLS), and zeta-potential (ZP) were performed to determine the crystal structure, effective hydrodynamic diameter, and surface potential, respectively, of the industrial and food grade TiO_2 particles. Crystal structure of TiO_2 particles was determined by scanning 2θ values from 10° to 70° for characteristic TiO_2 peaks. Hydrodynamic diameter (ZetaPALS, Brookhaven Instruments Corp.) was quantified via dynamic light scattering by suspending 120 ppm of TiO_2 in deionized water. Particles were then sonicated for 30 minutes before addition to respective medias where a final 30 second sonication was performed just before characterization. DLS and ZP (ZetaPALS) measurements were quantified in fresh colon medium before introduction to colon reactor, as well as, 10 mM

KCl and 191 mM solution representing equivalent ionic strength of the colon medium from pH range 4-8.

2.2.3 Phenotypic Microbial Characterization

Phenotypic characterization determines the systemic microbial community response to TiO_2 introduction combining genetic expression with conditions of the local environment. Testing was performed on the microbial community at the morning and afternoon feedings extracted from the colon fluid after being washed three times at 3,700 *x g* using 10 mM KCl. Bacterial cells were then suspended in 10 mM KCl for all phenotypic testing which consisted of cellular concentration, relative hydrophobicity, electrophoretic mobility (EPM), and protein and sugar content of extracellular polymeric substances (EPS). Cellular concentration was determined using a hemocytometer to quantify cells per volume (Haznedaroglu *et al.* 2012; Mihmanli *et al.* 2012; Gutierrez *et al.* 2016). Relative hydrophobicity was assessed by the microbial adhesion to hydrocarbon (MATH) test using n-dodecane as the model hydrocarbon allowing for the separation of bacteria with nonpolar surface groups from the aqueous phase. The higher the concentration of bacteria in the hydrocarbon phase, the greater the hydrophobicity value (Walker *et al.* 2005). EPM was quantified using Zeta-pals zeta potential analyzer utilizing an electric field to determine the relative surface potential of the microbiota in solution. EPS, as protein and sugar content bound to the microbial surface, was determined by following the freeze-dry method and quantified the biomacromolecules colorimetrically (Gong *et al.* 2009). These characterization tests were performed twice daily using methods published previously in greater detail (Marcus *et al.* 2013).

2.2.4 Biochemical Characterization

Biochemical characterization allowed for determination of the microbial community's response to the introduction of the two model ENMs. Specifically, variations in electrical conductivity and pH were observed to determine disturbances caused by the addition of ENMs to the system. Conductivity (YSI 3200 Conductivity Instrument Model #3200 115V; YSI, Yellow Springs, OH) and pH (Thermo Scientific Orion Star A214; Waltham, MA) were monitored twice daily during morning and afternoon sampling. Conductivity measurements allowed for indirect quantification of ionic strength of colon fluid (Griffin *et al.* 1973).

2.2.5 Microbial Community Composition

Microbial community composition determined shifts in the structure of the community resulting from system perturbations associated with ENM introduction. Samples for DNA sequencing were collected and stored at -20°C each day during the morning feeding. DNA extraction was performed using the MoBio (Carlsbad, CA) total microbial extraction kit following the manufacturer's protocols. After extraction, samples were shipped to the Research and Testing Laboratories (Lubbock, TX) for bacterial tag-encoded pyrosequencing using the 28F-388R primer. Sequences were first denoised and assigned operational taxonomic units using quantitative insights into microbial ecology (QIIME) software to allow taxonomic units to then be assigned.

2.2.6 Statistical Analysis

Repeated One-Way Analysis of Variance (ANOVA) was used to determine variation of phenotypic and biochemical parameters between testing conditions to account for temporal dependency of data. Tukey Honestly Significant Differences (HSD) was the post-hoc test used to determine between which testing conditions (e.g. baseline, industrial grade, and food grade exposures) the statistical significance determined by repeated measures ANOVA occurred. An alpha value of 0.05 (p-value <0.05) was utilized to determine statistical significance between testing parameters (e.g. pH, conductivity, etc) from TiO_2 exposures.

2.3 Results

2.3.1 Nanoparticle Characterization

Characterization of food and industrial grade TiO_2 allowed for the quantification of physical properties known to influence the human and environmental fate and transport of the particles. Characteristic XRD 2θ peaks for anatase and rutile crystal formations are 25° and 27°, respectively (Figure 2.1) (Thamaphat *et al.* 2008). XRD results indicate that both industrial and food grade TiO_2 contained a mostly anatase crystal structure. Specifically, industrial grade samples were comprised of 75% anatase and 25% rutile while food grade TiO_2 was 98% anatase and 2% rutile.

Hydrodynamic diameters and zeta potential of industrial and food grade TiO_2, as functions of IS are presented in Figure 2.2A and B, respectively. Food grade TiO_2 has a largely unchanged diameter in 10mM KCl ranging between 212 ± 6 nm to 315 ± 15 nm from pH 4-8 indicating stability under idealized conditions (Figure 2.2A). Industrial grade hydrodynamic diameter increases with pH in 10 mM KCl between the range of 252 ± 15 nm to 864 ± 48 nm meaning less stability and greater aggregation. Zeta-potential in 10 mM KCl reveals the iso-electric point (IEP) of the food grade particles is between pH 3 and 4 while industrial grade is closer to pH 6.8 (Figure 2.2B). Findings agree with previous TiO_2 characterization studies determining the IEP of this industrial grade TiO_2 as being between pH 6.2-6.8 (Chowdhury *et al.* 2011; Yang *et al.* 2014) and the food grade sample of approximately 3.5 (Yang et al., 2014). Food grade TiO_2 represents a far more stabilized particle under idealized solution conditions.

Additionally, hydrodynamic diameter and zeta-potential of both TiO_2 types were quantified at the equivalent ionic strength of colon medium (191 mM) using solely the colon media salts (NaCl, $CaCl_2$, K_2HPO_4, $MgSO_4$, and $FeSO_4$) dissolved into deionized water (Figures 2A and B). Figure 2A shows that the hydrodynamic diameters for both TiO_2 types undergo little change across pH ranges 4 to 8, however, industrial grade aggregates were far larger averaging ~1091 nm compared to ~330 nm with food grade samples. Industrial and food grade TiO_2 particles also exhibited similar zeta-potential values at the higher IS (191 mM) between pH 4 and 8 (industrial grade: -14.7 mV; food grade: -14.1 mV) in a solution containing both monovalent and divalent cations. Comparison between the dilute monovalent solution and the equivalent ionic strength of the colon medium in Figures 2.2A and 2.2B indicates that the stability associated with TiO_2 is mediated by the solution chemistry of the complex media. Zeta-potential values do indicate that double layer compression occurs with both particles as to be expected in the presence of divalent cations (e.g. Ca^{2+}) (Gregory 2004). However, differences in hydrodynamic diameter between the two TiO_2 types suggests that the food grade particles still exhibit a degree of stability limiting the extent of their aggregation in complex media.

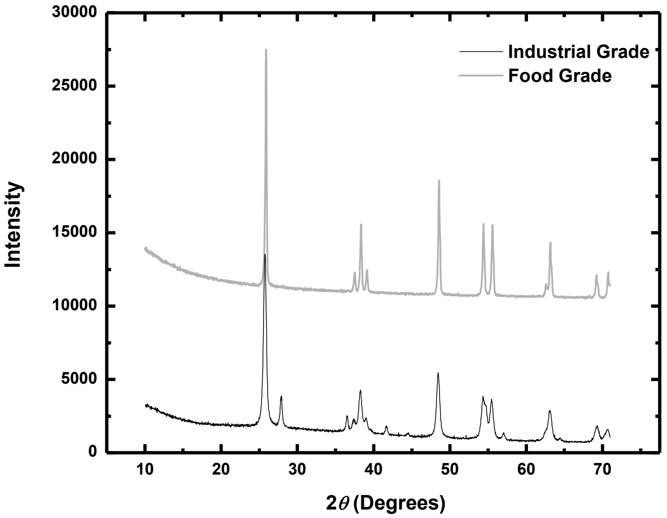

Figure 2.1: X-ray diffraction pattern of food grade TiO_2 with anatase and rutile peaks denoted by (A) and (R), respectively. Error bars represent the standard error of triplicate measurements.

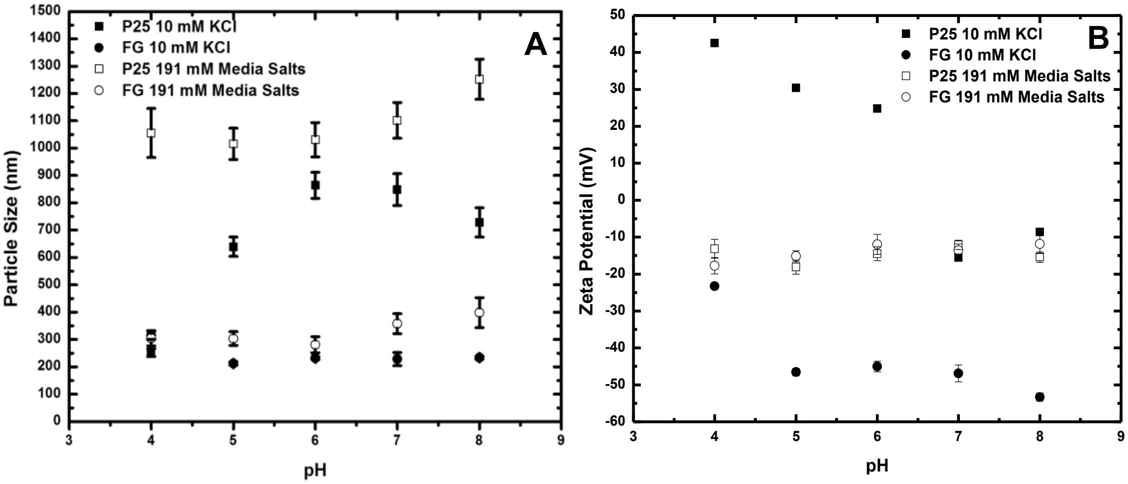

Figure 2.2: Variations in (A) particle size and (B) zeta potential (ZP) of 120 ppm TiO_2 between pH ranges 4 to 8. Industrial and food grade TiO_2 particles were introduced into 10 mM KCl as a model monovalent electrolyte solution and into 191 mM salt solution containing only the salts present in the colon medium (NaCl, $CaCl_2$, K_2HPO_4, $MgSO_4$, and $FeSO_4$). ZP values were calculated from electrophoretic mobility measurements using the Smoluchowski equation. Error bars represent the standard error of triplicate measurements.

2.3.2 Phenotypic Characterization

Cellular concentration was used to determine the quantity of bacterial cells present in colonic fluid (Figure 2.3A). Bacterial colonization of the intestines aids normal physiological processes, supports the immune system, and helps defend against pathogen growth (Reid et al. 2001; Hooper et al. 2002; Lupp et al. 2007). Gut microbiota concentration ranges from 10^3 to 10^{12} bacteria per mL with highest values observed within the colon (Xu et al. 2003; Posserud et al. 2006). Cellular concentration oscillated throughout the week during baseline conditions starting at $4.32 \pm 0.6 \times 10^9$, reaching a high of $6.06 \pm 1.9 \times 10^9$, and ending at a low of $3.47 \pm 1.01 \times 10^9$ by day five indicating values remained within a normal range during under control conditions. Both TiO_2 treatment conditions additionally resulted in decreases of cellular concentration during the five day exposures. An 80% reduction was observed from the starting cell concentration during industrial grade TiO_2 exposure (8.96 ± 2.4 to 1.78 ± 0.6, $\times 10^9$ cells/mL). Food grade TiO_2 exposures varied showing an initial increase in cellular concentration of approximately 20% during days 1 and 2; however, a total reduction of 58.6% (2.88 ± 0.5 to 1.19 ± 0.2, $\times 10^9$ cells/mL) was observed from the start to the end of the week. Overall, both TiO_2 exposures resulted in a larger decrease in cell concentration than the 19.6% reduction (4.32 ± 0.6 to 3.5 ± 1.0, $\times 10^9$ cells/mL) observed during baseline conditions suggesting potential inhibition of physiological processes (Reid et al. 2001). Differences observed in the cell concentration were not of statistical significance (P-value: 0.165, baseline: 4.5 ± 0.4, industrial grade: 5.2 ± 1.2, food grade: 2.5 ± 0.4, $\times 10^9$cells/mL).

Quantification of the microbial community's relative hydrophobicity provides insight into microbial aggregation, EPS production and biofilm formation (Schafer et al. 1998; Marcus et al. 2013). Microbial aggregates and biofilms in the human body are indicative of deleterious, inflammatory chronic conditions (e.g. cystic fibrosis, ulcerative colitis) and can prevent nutrient absorption by blocking the pili of the intestines (Fallingborg et al. 1993; Raisch et al. 2014; Johnson et al. 2015). Baseline conditions resulted in a gradual rise in hydrophobicity from 6 ± 6.3% on day 1 to 35 ± 6.5% by day 5 (Figure 2.3B). The trend of increasing relative hydrophobicity of the microbiota was also observed previously with a similar in vitro ENM investigation, although values were slightly lower than the current study (1–5% ± 0.8–5.3% to 34 ± 16.8 %) (Taylor *et al.* 2015). Exposure to industrial grade and food grade ENMs also resulted in increases from lows between 0-5% hydrophobicity to 34 ± 4.7% and 43 ± 4.1%, respectively, representing a range similar to that of the baseline conditions Relative hydrophobicity trended comparably to baseline conditions during food grade TiO_2 exposures until day 3 when baseline reduced from 35 ± 3.5% to 28 ± 2.4%, between days 3 and 4, while food grade increased from 39 ± 3.9% to 43 ± 4.1% for the same time point. Between all testing conditions, the greatest relative hydrophobicity was observed between days 3 and 4 of industrial grade TiO_2 exposures noted by an increase from 45.2 ± 6.1% to 58.6 ± 3.5%. After this increase, however, the relative hydrophobicity during industrial grade exposures returns to near baseline levels by day 5 (34.6 ± 4.6% and 35.2 ± 6.5%, respectively). Differences in relative hydrophobicity were statistically significant between baseline and food grade testing conditions as determined by repeated measures ANOVA (P-value: 0.005, baseline: 27.5 ± 5.5, industrial grade: 29.6 ± 11.3, food grade: 32.4 ± 7.1) and Tukey HSD values (HSD: 27, baseline and industrial grade: 2; baseline and food grade: 5; industrial and food grade: 3). While the conservative nature of Tukey HSD is not always powerful enough to capture statistical significance between means it can suggest between which testing conditions the statistical significance occurs (Jaccard et al. 1984; Vasey et al. 1987). General trends in relative hydrophobicity indicate a slightly more hydrophobic microbiota in the presence of both TiO_2 types than baseline conditions with food grade exposures slightly higher than that of industrial grade exposure.

Electrophoretic mobility (EPM) provides a relative measure of the surface charge of the microbiota which is linked to bacterial aggregation and microbial community stability; where higher absolute EPM values are linked with more stable microorganisms (Hermansson 1999; Marcus et al. 2013). EPM during baseline and both TiO_2 exposures show similar trends of increasing negativity of the microbial community until day 4 when the trends begin to deviate (Figure 2.3C). Baseline conditions became more negative (higher absolute value) by day 5; whereas, both TiO_2 exposures resulted in reduced negativity (lower absolute value) (baseline: -1.61 ± 0.1, industrial grade: -1.5 ± 0.1, food grade: -1.3 ± 0.03, $[(\mu m\ s^{-1})/(V\ cm^{-1})]$), respectively). However, although baseline conditions are more negative than both TiO_2 exposures by day 5, the EPM of the microbiota between days 2 through 4 was slightly more negative in the presence of TiO_2 particles suggesting greater microbiota stability during this time. Microbiota EPM during baseline conditions (-1.61 ± 0.1 $[(\mu m\ s^{-1})/(V\ cm^{-1})]$) reflect a similar value (-1.67 ± 0.2 $[(\mu m\ s^{-1})/(V$

cm^{-1})]) as control values obtained in a previous study utilizing a similar experimental design (Marcus et al., 2013). Variations observed in the EPM were not of statistical significance as determined by repeated measures ANOVA (P-value: 0.063, baseline: -1.4 ± 0.2, industrial grade: -1.44 ± 0.2, food grade: -1.5 ± 0.1) which suggest similar microbial aggregation and transport behavior during each testing condition.

The sugar and protein content of EPS produced by the microbiota are indicators of cellular conditions around the microbial community (Eboigbodin et al. 2008). Sugar content of the EPS during baseline conditions remained mostly constant towards midweek (36.4 ± 3.7 to 43.6 ± 4.3, mg/10^{10} cells), when values further reduced by day 5 (26.82 ± 10.7, mg/10^{10} cells) (Figure 2.4A). Relatively constant EPS sugar content produced by the microbiota was also observed previously during control conditions although values were much lower (3.6 ± 0.2mg/cell·10^{10}) (Taylor *et al.* 2015). Industrial grade exposure resulted in a similar starting sugar content (29.7 ± 3.4 to 46.36 ± 6.1, mg/10^{10} cells) as baseline conditions through day 3, prior to an end of the week spike at 117.8 ± 23.5 mg/10^{10} cells (~300% increase over five days). Conversely, food grade exposures resulted in mostly constant and lower sugar content in the EPS (11.6 ± 0.7 to 13.5 ± 3.7, mg/10^{10} cells) than both industrial grade and baseline conditions during days 1-4 and increased slightly higher than baseline on day 5 (46.6 ± 16.9 and 26.8 ± 10.7, mg/10^{10} cells, respectively). Differences observed in the sugar content were not of statistical significance as determined by repeated measures ANOVA (P-value: 0.253, baseline: 34 ± 3.5, industrial grade: 57 ± 16, food grade: 19 ± 7, mg/10^{10} cells, respectively).

Protein content of the EPS produced by the microbiota increased gradually until day 3 from 177.9 ± 17 to 240.5 ± 59, mg/10^{10}, cells during baseline conditions (Figure 2.4B). After midweek, the protein content then decreased to 121.72 ± 44 mg/10^{10} cells before rising sharply to 325.8 ± 119 mg/10^{10} cells by day 5. Increasing protein content of the EPS produced by the microbiota was also observed previously with a similar in vitro ENM investigation, although values were lower than the current study (40-80 mg/10^{10} cells) (Taylor et al. 2015). Protein content of the EPS during both TiO_2 exposures followed a similar trend observed during baseline conditions remaining higher with food grade exposures and lower with industrial grade exposures. Differences observed in the protein content between testing conditions were of statistical significance as determined by repeated measures ANOVA (P-value: 0.0004, baseline: 213 ± 34, industrial grade: 207 ± 49, food grade: 290 ± 52). Tukey HSD values for protein content (HSD: 110, baseline and industrial grade: 6; baseline and food grade: 77; industrial and food grade: 83.), while conservative, still support that the statistical significance is observed between food grade exposures and both the industrial and baseline conditions. This difference is also evident in the trends observed in Figure 2.4B.

The sugar to protein ratio (S/P ratio) is utilized as an indicator of cellular conditions, whereas increased sugar content suggests microbial aggregation and biofilm formation (Marcotte et al. 2007). During baseline conditions, the S/P ratio increased from day 1 to 4 by ~140% (0.24 ± 0.04 to 0.57 ± 0.1, respectively) followed by a sharp reduction on day 5 to 0.09 ± 0.01 (Figure 2.4C). Industrial grade exposures follow baseline conditions early in the week as the S/P ratio nearly doubles from 0.243 ± 0.044 on day 2 to 0.424 ± 0.046 by day 4 and rises slightly higher into day 5 (0.489 ± 0.088) contrasting with baseline observations. Alternatively, food grade exposures resulted in a largely unchanged S/P ratio throughout the week with the exception of day 5 where an increase of 40% (0.05 to 0.07) occurred from the start of the week. Variations observed in the S/P ratio between testing conditions were not of statistical significance as determined by repeated measures ANOVA (P-value: 0.58, baseline: 0.327 ± 0.184, industrial grade: 0.357 ± 0.1, food grade: 0.057 ± 0.011). However, Tukey HSD values for S/P ratio show near statistical significance between group means of testing conditions with food grade exposures in relation to both industrial and baseline conditions which is also supported by trends in Figure 2.4C; although, as values remain just below 0.4, they are not reflected as significant by this conservative procedure (HSD: 0.4, baseline and industrial grade: 0; baseline and food grade: 0.3; industrial and food grade: 0.3).

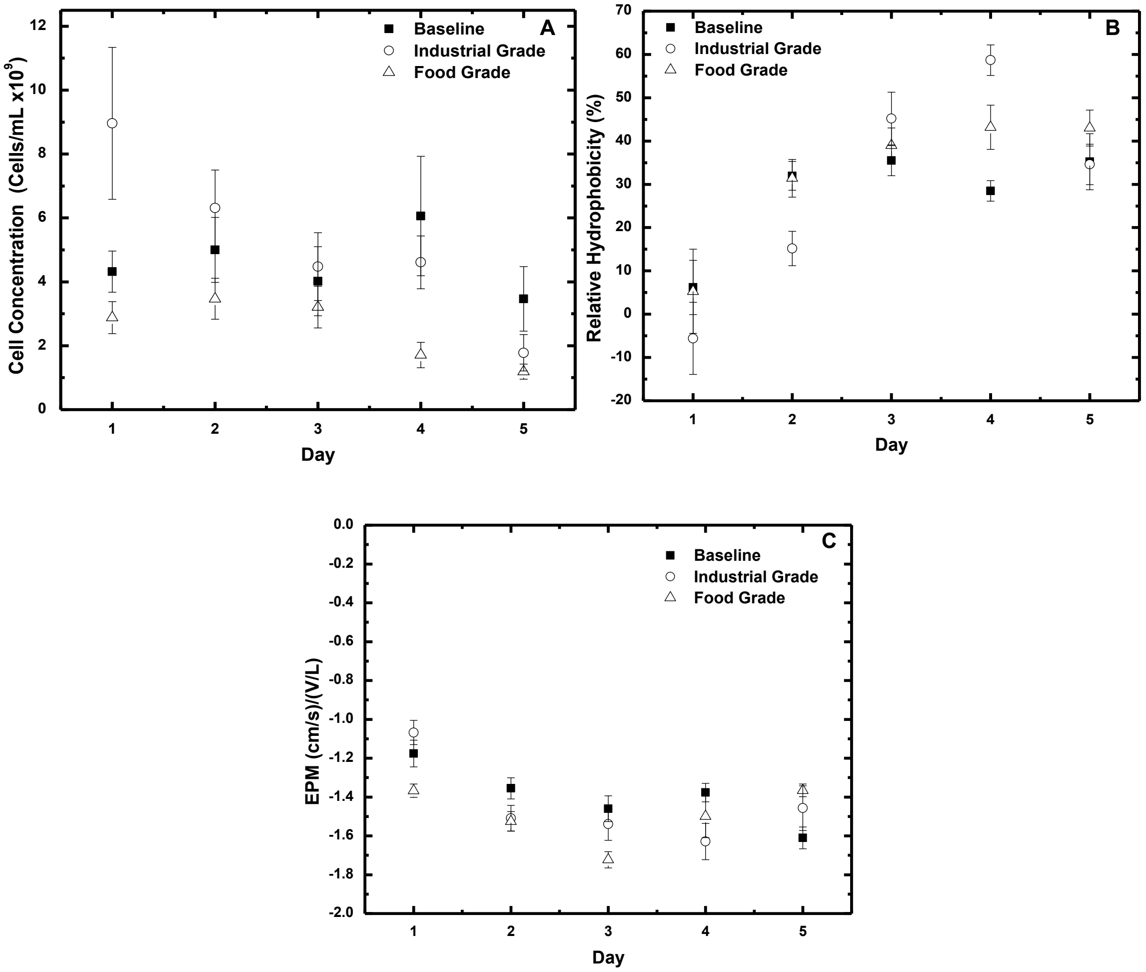

Figure 2.3: Changes in microbial (A) cellular concentration, (B), relative hydrophobicity, and (C) electrophoretic mobility (EPM) in a model colon reactor measured daily over a 5-day experiment. Conditions tested include exposure to 36 mg/L/day industrial grade or food grade TiO_2 nanoparticles and the particle free control (referred to as baseline). Error bars represent the standard error of triplicate measurements of daily averages between reactor runs of the same experimental condition.

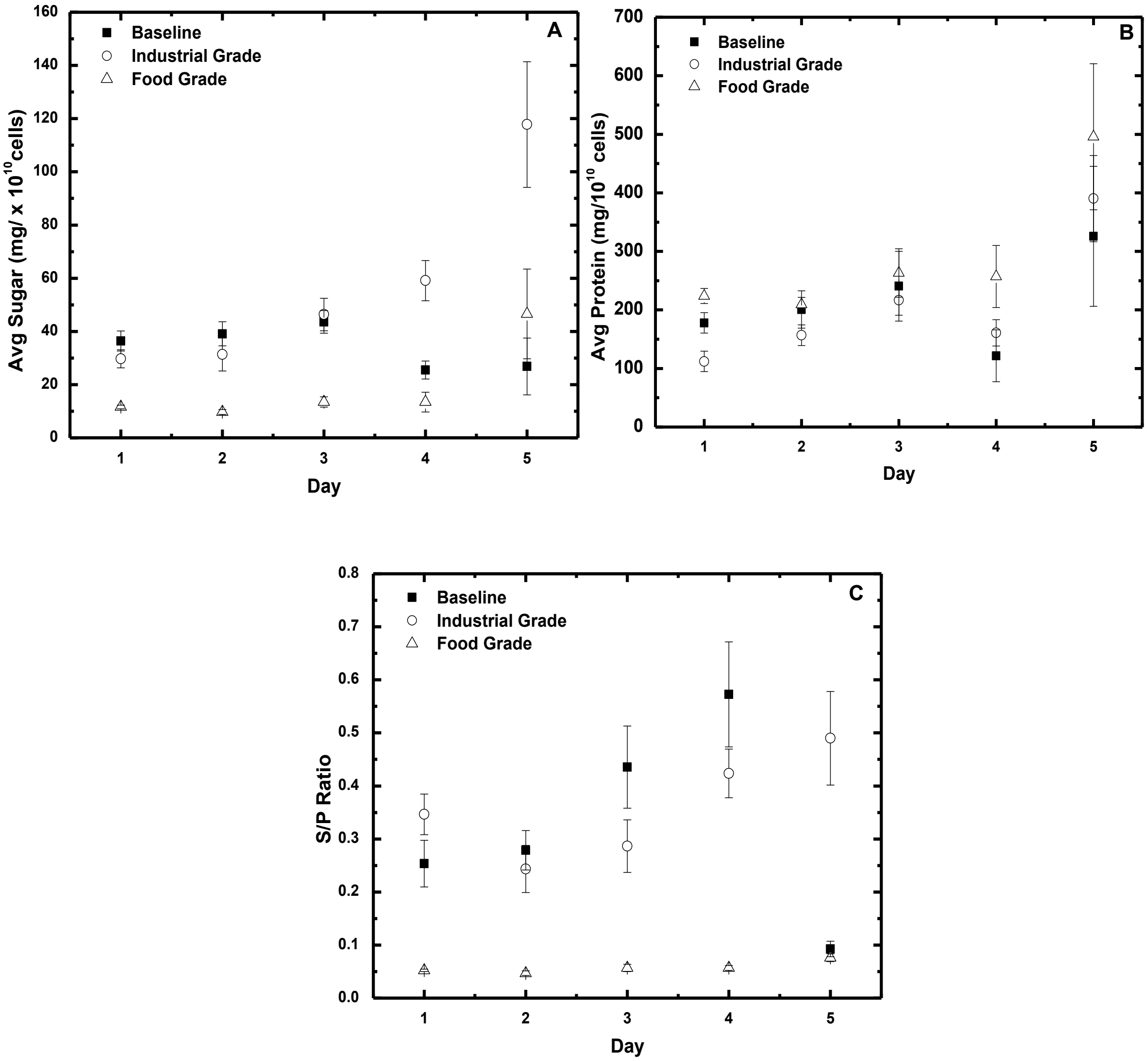

Figure 2.4: Variations in average (A) sugar content and (B) protein content produced by the human gut microbiota in model colon reactor, as well as the (C) sugar to protein ratio observed over the 5-day experiment. Conditions tested include exposure to 36 mg/L/day industrial grade and food grade TiO_2 nanoparticles and the particle free control (referred to as baseline). Error bars represent the standard error of triplicate measurements of daily averages between reactor runs of the same experimental condition.

2.3.3 Biochemical Characterization

Measurement of colonic fluid pH provides insight into microbial degradation of energy sources and overall colon health (Nugent et al. 2001). Healthy colonic pH values typically fall between 5.5 and 7.5 (Nugent et al. 2001). Colonic pH during baseline conditions generally remains in this range decreasing from 6.7 ± 0.1 to 5.4 ± 0.1 from day 1 to 5 with a weekly low occurring on day 3 of 4.9 ± 0.1 as shown in Figure 2.5A. Industrial grade exposure also resulted in a pH reduction from 6.7 ± 0.03 to 4.7 ± 0.1 from day 1 to 5. Food grade exposures additionally decreased reaching the lowest pH of all testing conditions by day 5 (6.4 ± 0.05 to 4.1 ± 0.02). Reductions in intraluminal colonic pH suggests significant changes occurring within the colon possibly indicating the onset of deleterious colonic conditions (Nugent et al. 2001). The pH recovery observed on day 3 in baseline conditions shifted towards day 4 (4.5 ± 0.1) with industrial grade exposures and day 5 (4.1 ± 0.02) for food grade exposures. Variations observed in the pH between testing conditions were of statistical significance as determined by repeated measure ANOVA (P-value: 0.0004, baseline: 5.5 ± 0.8, industrial grade: 5.2 ± 0.4, food grade: 4.9 ± 0.4). Tukey HSD values for pH indicate that none of the statistically significant variance in the data set is reflected by the conservative estimates for significance amongst group mean values (HSD: 0.9, baseline and industrial grade: 0.2; baseline and food grade: 0.6; industrial and food grade: 0.3). However, HSD values do suggest that the statistical significance quantified using ANOVA likely lies between food grade exposures and baseline conditions as the 0.6 difference is nearest to the 0.9 HSD value.

Conductivity measurements quantify ionic content of the solution as well as indirectly approximate ionic strength (Griffin et al. 1973). Short chain fatty acids (SCFAs) are organic anions produced from the degradation of organic compounds within the human colon partially responsible for increasing ionic content (Nugent *et al.* 2001) The production and further degradation of SCFAs alters the conductivity of the intraluminal colonic fluid which would additionally be reflected in the system pH. Baseline conditions recorded an increase from day 1 to 2 of 4.7 ± 0.3 μS cm^{-1} to 5.5 ± 0.5 μS cm^{-1} before reducing to 5.1 ± 0.4 μS cm^{-1} on day 3 and remaining largely constant into day 5 (Figure 2.5B). Findings suggest mineralization of the organic compounds is indeed occurring during the control weeks. The conductivity of the matrix upon industrial grade particle exposure was higher on Day 1 (6.8 ± 0.3 μS cm^{-1}) than baseline conditions prior to reaching comparable levels on day 2 (industrial grade: 5.3 ± 0.2; baseline: 5.5 ± 0.5 μS cm^{-1}). These values remained higher increasing to 6.4 ± 0.3 μS cm^{-1} by day 5. Conductivity during food grade exposure contrasts with industrial grade exposures by remaining relatively constant showing only a 6% increase from beginning to end of the week. Notably, the highest value of conductivity was observed on day 4 during industrial grade exposure measuring 38% and 50% greater than baseline conditions and food grade exposure, respectively.

Variations observed between testing conditions in conductivity were not of statistical significance as determined by repeated measures ANOVA (P-value: 0.63, baseline: 5.092 ± 0.1, industrial grade: 6.332 ± 0.3, food grade: 4.59 ± 0.09). However, Tukey HSD values do indicate statistical significance between group means for industrial and food grade conditions (HSD: 1.4; industrial and food grade: 1.7) and nearly significant between baseline and industrial grade (HSD: 1.4; baseline and industrial grade 1.2) which is supported by trends observed in Figure 2.5B. Findings indicate that industrial grade TiO_2 exposure may increase the production of acidic metabolites, reflected by the highest conductivity, whereas the food grade TiO_2 exposures conversely result in an inhibitory action determined by lower conductivity than baseline conditions.

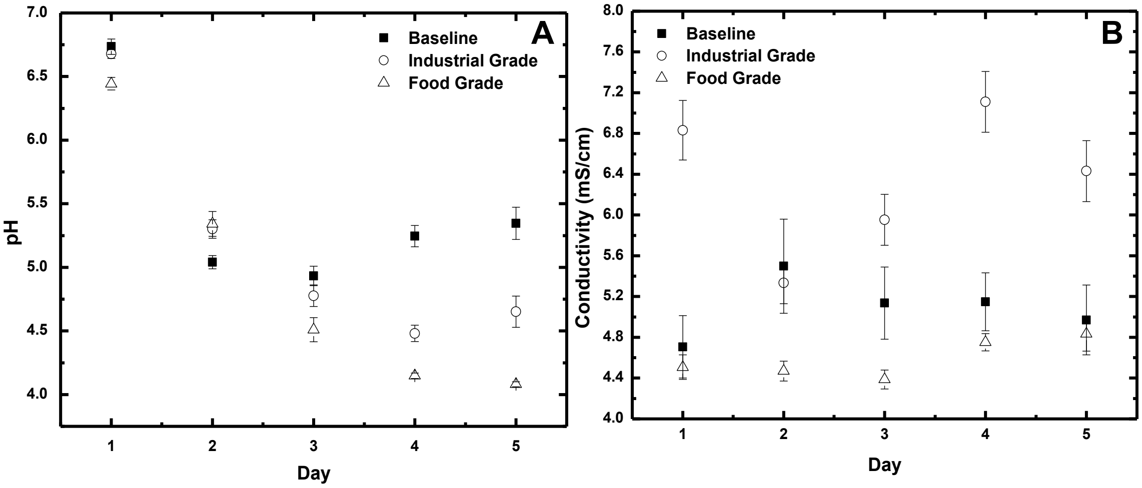

Figure 2.5: Changes in system (A) pH and (B) conductivity in a model colon reactor inoculated with a human gut microbiota measured daily over a 5-day experiment. Conditions tested include exposure to 36 mg/L/day industrial grade or food grade TiO_2 nanoparticles and the particle free control (referred to as baseline). Error bars represent the standard error of triplicate measurements of daily averages between reactor runs of the same experimental condition.

2.3.4 Microbial Community Composition

Quantification of the microbial community allows for the characterization of the most abundant bacteria within the gut microbiome (Arumugam et al. 2011; Marcus et al. 2013). *Proteobacteria* dominance on day 1 (99 ± 0.0 %) in baseline conditions and both TiO_2 exposures indicates a consistent microbial distribution at the start of each experiment (Figure 2.6). *Firmicutes* became the most abundant phyla by day 5 under baseline conditions accounting for 81± 0.0 % of the microbial community. Under exposure to industrial grade particles, a greater combination of phyla existed. Notably, *Proteobacteria* initially dominated at 99 ± 0.0 %, but reduced to 32 ± 0.0 % while *Firmicutes* and *Bacteroidetes* became more abundant (46 ± 0.2 % and 22 ± 0.2 %, respectively). Alternatively, food grade exposure resulted in *Proteobacteria* decreasing (99 ± 0.0 % to 86 ± 0.0 % over five days), but remaining the most abundant phyla present. Only minor increases were observed for both *Firmicutes* and *Bacteroidetes* from 1.3 ± 0.0 % to 11 ± 0.0 % and 0.234 ± 0.0 % to 2 ± 0.0 %, respectively. Increased quantities of *Firmicutes* compared to *Bacteroidetes* in the microbiota have been associated with increased energy reabsorption and obesity (Ley et al. 2005; Ley et al. 2006; Turnbaugh et al. 2006).

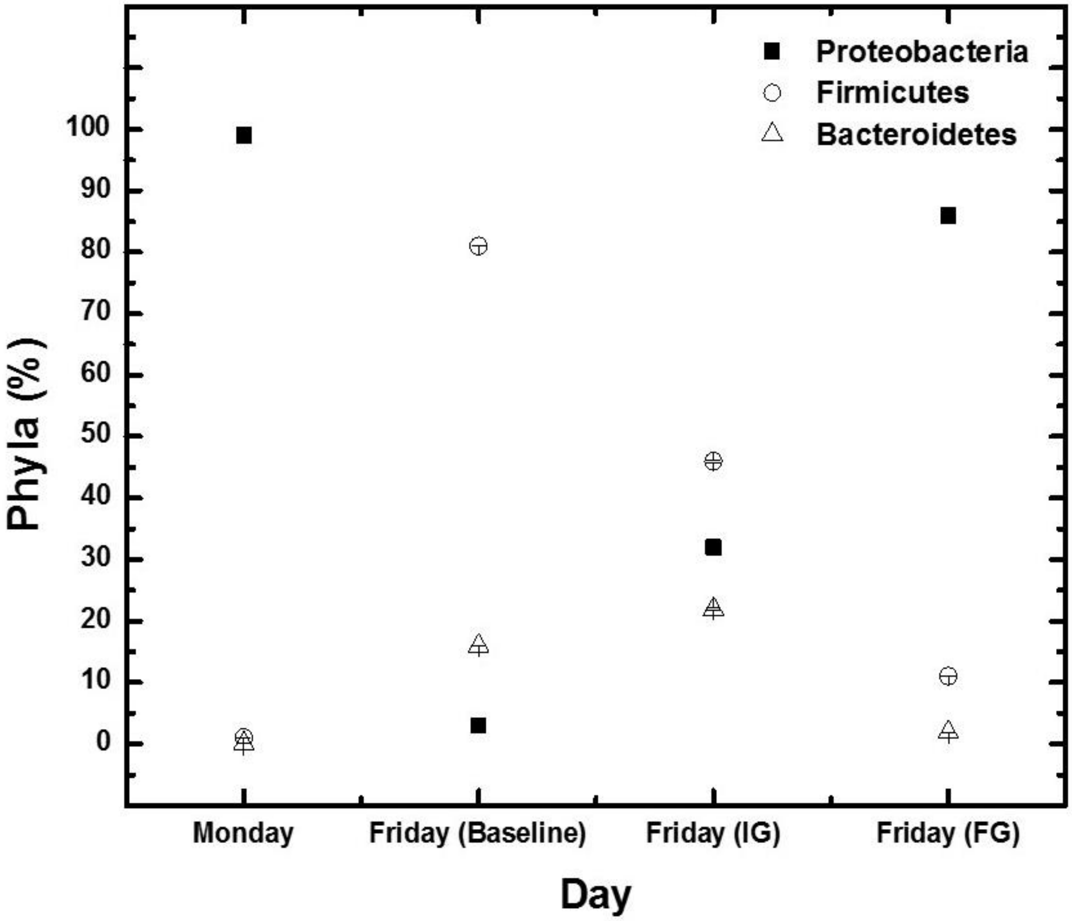

Figure 2.6: Changes in microbial composition of the human gut microbiota in a model colon reactor measured on day 1 (Monday) and day 5 (Friday) during a 5-day experiment. Conditions tested include exposure to 36 mg/L/day industrial grade (IG) or food grade (FG) TiO_2 nanoparticles and the particle free control referred to as baseline (BL). Monday represents all three testing conditions since each began with same starting microbial composition (Proteobacteria > 99 ± 0.0 %). The microbial compositions observed on Friday are presented individually for each testing condition. Error bars represent the standard error of triplicate measurements of daily averages between reactor runs of the same experimental condition.

2.4 Discussion

2.4.1 Microbial Community Composition

Assessment of the microbiota in the absence of TiO_2 particles (baseline conditions) revealed that the community naturally shifted from a phyla composition dominant in *Proteobacteria* to that of *Firmicutes* over the course of the five-day experiment. Numerous studies determined that the human gut microbiota largely consists of *Bacteroidetes* in *in vitro* models and *Firmicutes* in real gut microbiomes when fed diets high in proteins and fats (Ley *et al.* 2006; Turnbaugh *et al.* 2006; Zoetendal *et al.* 2008; Arumugam *et al.* 2011). While the *Proteobacteria* dominance observed at the start of each experimental condition conflicts with the reported compositions, this can be attributed to the microbiota donor consuming a vegetarian diet with greater amounts of carbohydrates rather than animal proteins and fats (Arumugam *et al.* 2011). Further, studies have determined that long term dietary selection plays a major role in the normal composition of the microbial community (Gill *et al.* 2006; Benson *et al.* 2010). However, in addition to this, sudden changes in diet can cause rapid community shifts to occur supporting the phyla shift observed in the microbiota during baseline conditions (Wu *et al.* 2011; David *et al.* 2014). Moreover, Turnbaugh et al., (2009) utilized metagenomics analyses to observe and report a shift in microbial composition to *Firmicutes* dominant in humanized gnotobiotic mice when the diet was switched from mouse chow to a western diet (Turnbaugh *et al.* 2009). Yet even with perturbations, the gut microbiota, overall, is quite stable with consistent diet and capable of restoration after perturbations (Vanhoutte *et al.* 2004; Costello *et al.* 2009). Therefore, a diet high in animal protein and fats as commonly found in western diets explain the microbial shift from *Proteobacteria* to *Firmicutes* observed during these control conditions.

Interestingly, inhibition of the baseline transition in microbial composition from *Proteobacteria* to *Firmicutes* dominant (as anticipated via dietary considerations) occurs during TiO_2 exposures. Food grade TiO_2 exposures only show a minor drop in *Proteobacteria* from 99 ± 0.0 % to 86 ± 0.0 % indicating a largely unchanged microbiota with this form of TiO_2. *Proteobacteria* during industrial grade exposure was reduced from 99 ± 0.0 % to 32 ± 0.0 %, however, the *Firmicutes* dominance was less than that observed during baseline conditions (46 ± 0.2 % and 81 ± 0.0 %, respectively). Microbial composition is of vital importance as the human gut microbiota plays a significant role in maintaining colonic health and preventing the development of disease (Sekirov et al. 2010). Increased quantities of *Firmicutes* compared to Bacteroidetes in the microbiota are associated with increased energy reabsorption and obesity (Ley et al. 2005; Ley et al. 2006; Turnbaugh et al. 2006). Thus, the greatest energy reabsorption may occur normally during baseline conditions as *Firmicutes* composition dwarfs Bacteroidetes (81 ± 0.0 % to 16 ± 0.0 %, respectively) compared to both TiO_2 exposures with the least energy reabsorption possibly during food grade exposure (*Firmicutes*: 11 ± 0.0 %; Bacteroidetes 2 ± 0.0 %, respectively).

Differences in microbial community composition may be explained by direct interaction between TiO_2 particles and the bacteria (Jiang et al. 2009; Chowdhury et al. 2012) , as well as, binding of colon medium molecules to the TiO_2 surfaces (Cedervall et al. 2007; Jiang et al. 2010; Walkey et al. 2012). Either binding action (bacteria and TiO_2; colon medium and TiO_2) can result in aggregation that prevents the microbiota from reaching the energy sources most necessary to remain viable. Microbial aggregates are associated with increased biofilm within the human colon which has been linked to the development chronic, inflammatory conditions (Fallingborg et al. 1993; Raisch et al. 2014; Johnson et al. 2015). As such, the hindered community transition in presence of TiO_2 particles from *Proteobacteria* to *Firmicutes* dominant may potentially be due to surface interactions preventing access to microbial nutrients potentially resulting in persistent, colonic health concerns.

Particle stability is known to influence the fate and transport of nanoparticles and depends upon the surface and solution chemistries. Electrophoretic mobility (used to calculate zeta-potential (Gregory 2004) provides a measure of ion accumulation around the surface for assessing particle stability. These properties impact the capacity of the particles to approach and interact with other particles leading to homo-aggregation. Previous work with food and industrial grade TiO_2 suggest that the aggregation and stability are sensitive to the pH of the solution relative to the isoelectric point (IEP) of the particle (Zhang et al. 2008; Domingos et al. 2009; French et al. 2009; Keller et al. 2010; Chowdhury et al. 2011; Yang et al. 2014). Notably, industrial grade particles have an IEP near pH 6.8 and food grade closer to pH 3.5 (Yang et al., 2014). DLS and ZP measurements shown in Figures 2.2A and B support that the IEP for industrial and food grade TiO_2 occurs near pH 6.8 and < 4, respectively, using both 10 mM KCl, as well as, the salts present in the colon medium (ionic strength 191 mM). The intial pH of the colon media at particle introduction (~6.7) suggests greater electrostatic stability with food grade particles than the industrial grade form of TiO_2 likely resulting from an intentionally added phosphate surface coating for enhanced stability during food prepartion and consumption (Yang et al. 2014). Further, previous work determined that no detectable quantity of stabilizing agents (i.e. phosphorus coating) were present with industrial grade samples (Yang et al. 2014). The lack of surface enhancements and an IEP near the initial colon medium pH (~6.4- 6.8) supports that industrial grade TiO_2 would be unstable in this system, and therefore more prone to aggregation with other TiO_2 particles and solids in the colon media. Alternatively, stable food grade TiO_2 particles could also explain the lack of shift in microbial composition resulting from limited interaction between the negatively charged TiO_2 particles and microbial surfaces.

2.4.2 Biochemical Characterization

Particle aggregation may partially explain the limited microbial community shift during TiO_2 exposure; however, it does not entirely address other observations such as with conductivity measurements (Table 2.1 and Figure 2.5B). Comparison of only colon medium salts in deionized water with actual colon media (without bacteria and TiO_2 particles) shows similar conductivities corresponding to 191 mM (11.4 ± 0.0 mS/cm and 11.2 ± 0.1 mS/cm, respectively) suggesting that the initial conductivity results from salts and variations likely indicate acidic metabolite production, or the lack there of, within the colon reactor (Table 2.1). Short chain fatty acids (SCFAs) are weak acids, pKa near 4.8, meaning they exist as organic anions under these normal conditions (Nugent et al. 2001) and increases would indicate greater production based on the performed conductivity controls. At the same IS (191 mM media salts in DI water), industrial and food grade TiO_2 particles resulted in slightly lower conductivity than the control of 11.4 ± 0.0 mS/cm (9.0 ± 0.2 mS/cm and 9.4 ± 0.2 mS/cm, respectively). Within the actual colon media, the conductivities of the control (without particles and bacteria), industrial grade, and food grade are 11.2 ± 0.1 mS/cm, 10 ± 0.1 mS/cm, and 11 ± 0.5 mS/cm, respectively. These values reveal that both types of TiO_2 are capable of altering the ionic content of the solution; however, this effect is reduced when particles are introduced into the colon media.

Ionic Strength	Control (mS/cm)	Industrial Grade (mS/cm)	Food Grade (mS/cm)
95 mM	5.7 ± 0.2	4.2 ± 0.1	4.6 ± 0.1
191 mM	11.4 ± 0.0	9.0 ± 0.2	9.4 ± 0.2
Colon Media (191 mM)	11.2 ± 0.1	10.0 ± 0.1	11.0 ± 0.5
380 mM	14.7 ± 0.3	17.1 ± 0.4	16.6 ± 0.4

Table 2.1: Conductivity of 120 ppm industrial and food grade TiO_2 at varied ionic strengths (IS). Industrial and food grade TiO_2 particles were introduced into 95 mM, 191 mM, and 380 mM IS solution only the salts present in the colon medium (NaCl, $CaCl_2$, K_2HPO_4, $MgSO_4$, and $FeSO_4$). Additionally, both particles were added into fresh colon media including carbohydrates and proteins (influent) at an equivalent IS of 191 mM for comparison to controls. Standard error of triplicate measurements is presented.

System conductivity (after passing through colon) results suggest higher ionic content within the colon in the presence of industrial grade particles as compared to baseline conditions and food grade TiO_2 exposure (Figure 2.5B).Greater conductivity (suggesting higher ionic strength) is anticipated to result in increased aggregation of destabilized industrial grade particles with molecules in the colon medium (French et al. 2009). Derjaguin-Landau-Verwey-Overbeek (DLVO) theory supports the concept that electrical double layer compression occurs with increasing ionic strength across IS ranges of 1-100 mM (Chowdhury et al. 2011). While electrostatic differences between the three testing conditions may be minimal, any increase or reduction in ionic strength is still theoretically expected to alter the electrostatic forces influencing system interactions.

Lower conductivity observed with baseline conditions and the stable food grade particles may suggest the occurrence of a more comparable shift in microbial composition between the two as food grade particles and the microbial community both possess negative surface charges indicative of electrostatically repulsive conditions (Figure 2.2B and Figure 2.3C, respectively). Yet, this was not the case (Figure 2.6). Rather, the major shift observed during baseline conditions from *Proteobacteria* (>99% reducing to 3%) to *Firmicutes* (<1% increasing to 81%) dominant while food grade conditions remain largely unchanged (13% reduction in *Proteobacteria* (99% to 86%) suggests that the phosphate coating applied to stabilize food grade particles may not prevent interactions with molecules in the complex colon medium.

Proteins in complex solutions have been shown to cause particles to destabilize and aggregate in conditions similar to this study (Cedervall *et al.* 2007; French *et al.* 2009). Cationic binding between proteins and the stable, negatively charged food grade particles is one such possible mechanism that can occur within complex solutions of the human body (Nel et al. 2009). Partial support of cationic binding can be observed from an idealized study where the presence of anionic surface groups (e.g. inorganic phosphate) on the food grade particles resulted in greater absorption of the cationic dye, methylene blue, than industrial grade TiO_2 at near neutral conditions (pH 6.6) (Yang et al. 2014). However, solutions containing proteins, carbohydrates, and other molecules with uneven charge distributions complicates the assumption that net positive molecules will attract net negative as negatively charged particles can bind to positive segments of the overall negatively charged molecule (Nel et al. 2009; Hamad-Schifferli 2013). As such, although both TiO_2 particles are expected to be coated in part with protein and other small molecules of the solution (Cedervall et al. 2007; Walkey et al. 2012; Hamad-Schifferli 2013), system conductivity and microbial composition suggests that food grade's stabilizing surface enhancements (Weir et al. 2012; Yang et al. 2014) may be responsible for greater interaction with substrates in the complex media that limit the possibility for an extensive community shift to occur as observed during baseline conditions.

Buffering of colonic pH resulting from microbial degradation of substrates that appears during baseline conditions seems to be impaired when TiO_2 particles are present within the system (Figure 2.5A). All three testing conditions recorded reductions in pH between days

1 and 3 before deviations between baseline conditions and TiO_2 exposures, as well as, between the TiO_2 exposures themselves occur. Natural decreases in pH within the human colon have been previously observed and associated with the fermentation of carbohydrates by the microbiota producing short chain fatty acids (Vernia et al. 1988). Decreases in colonic pH are followed by the mineralization of the short chain fatty acids by acetogens and methanogens in tandem with mucosal secretions of bicarbonate caused a pH recovery resulting in a higher intraluminal colonic pH (Nugent et al. 2001). This suggests that the trend of pH decrease and then recovery observed during baseline conditions is the response expected under these conditions. Delayed recoveries in pH during TiO_2 exposures between days 4 and 5 for industrial grade exposures and food grade TiO_2 exposure appearing to show no sign of a recovery indicate disturbances to the SCFA production and mineralization occur during TiO_2 presence. Deleterious, chronic conditions are associated with decreases of intraluminal colonic pH. Low or reduced colonic pH (2.2-4.7) has been linked with the onset of inflammatory diseases like ulcerative colitis and Crohn's disease (Fallingborg et al. 1989; Raimundo et al. 1992). These findings suggest that the introduction of both TiO_2 types may lead to unhealthy colonic conditions over time, with food grade TiO_2 exposures potentially having the slightly greater impact.

2.4.3 Phenotypic Characterization

Phenotypic characterization — EPS S/P ratio, EPM and relative hydrophobicity — of the human gut microbiota further captures the impacts of food grade and industrial grade TiO_2 exposures. Biofilm is produced under certain conditions as a stress response to protect bacteria from hostile environments(Costerton et al. 1999). Bacteria within the biofilm are shielded from antimicrobial agents and cellular desiccation by a hydrated layer surrounding the microbial community (Sutherland 2001). EPS plays a major role in microbial aggregation and biofilm production (Flemming 1989; Burdman et al. 2000; Tsuneda et al. 2003) with increased sugar content being linked to greater aggregation and biofilm development (Marcotte et al. 2007). Both industrial grade exposure and baseline conditions indicate increasing sugar content within the EPS compared to protein; whereas, protein dominance during food grade TiO_2 is suggested by the largely unchanged S/P ratio (Figure 2.4C). Conditions during food grade exposures may prove more tolerable to the initial gut microbiota; however, as EPS production is also dependent on nutrient availability, it could also be that conditions are unfavorable for biofilm development (Sutherland 2001). Generally, less microbial EPS production and biofilm formation is anticipated in the presence of food grade TiO_2 particles compared to industrial grade particles and baseline conditions.

EPM trends suggest that similar microbial community aggregation and transport can be expected in the presence or absence of the TiO_2 particles (Figure 2.3C). Nanoparticles coating the bacterial surface have been suggested as one mechanism that may alter the EPM of the microbial community (Jiang et al. 2009). However, comparable trends in microbial EPM between all test conditions may suggest that particles have minimal direct contact with the microbial community. Further, many studies report that nanoparticles tend to develop coatings of protein or other organic substances, therefore the minor variations in microbiota EPM may simply result from responses to colonic conditions and not necessarily particles coating the microbial surface (Cedervall et al. 2007; Walkey et al. 2012). The relatively close ratio of bacteria to TiO_2 particles within this study (~10^9 bacteria to 3.33 x10^{10} TiO_2 per mL of colon medium) if particles were well dispersed also supports that direct interaction between particles and bacterial surfaces is unlikely. DLS and zeta-potential values presented in Table 2.2 show aggregation is quite likely occurring between particles and colon medium as particle size increases during industrial grade exposure and food grade shows signs of steric hindrance (Chowdhury et al. 2013).

	Particle Size (nm)	Zeta Potential (mV)
Baseline	1480 ± 103	-14.9 ± 9.2
Industrial Grade	1723 ± 80	3.4 ± 10
Food Grade	1009 ± 57	2.8 ± 6.7

Table 2.2: Particle size and zeta potential of 120 ppm industrial and food grade TiO_2 in fresh colon medium. Zeta-potential values were calculated from electrophoretic mobility measurements using the Smoluchowski equation. Standard error of triplicate measurements is presented.

Relative hydrophobicity of the microbial community increased in each testing condition across the experimental week (Figure 2.3B). Biofilm has been shown to line the mucosal surface and can indicate disruption of the normal colon mucous barrier (Probert et al. 2002; Shah et al. 2008). The influence of TiO_2 in this process is considered negligible as similar trends exist for microbial hydrophobicity in the control and other experimental conditions (Figure 2.3B). Increases in hydrophobicity observed within this system can be attributed to lowered pH altering functional groups at microbial surface. Additionally, solutions possessing a higher ionic strength result in electrical double layer compression of the charged surface functional groups exposing a more hydrophobic surface (Yee et al. 2000; Abu-Lail et al. 2003). A prior study utilizing the same in vitro model colon similarly recorded increases in hydrophobicity in the presence of three model ENMs (CeO_2, TiO_2, and ZnO) during the five day experimental runs (Taylor et al. 2015). Microbial relative hydrophobicity of the current study indicate TiO_2 exposures may result in comparable microbial deposition to baseline conditions, possibly along the mucosal surface within the actual human colon.

This study assessed impacts to the human gut microbiome within an in vitro model colon from the addition of two different types of titanium dioxide particles (industrial grade and food grade). TiO_2 exposure resulted microbial compositional, phenotypic, as well as, biochemical changes within the colonic system. Notably, TiO_2 exposure was linked to reduced transition of the microbial community from *Proteobacteria* abundance to *Firmicutes* with this effect being more pronounced during food grade TiO_2 exposures.

Additionally, reduced system pH and conductivity were observed during food grade TiO_2 exposure suggesting disruption of the anaerobic digestive process. Production of lactate rather than short chain fatty acids can be attributed to this possibly suggesting the potential onset of deleterious conditions over continuous, long term exposure. Interestingly, while protein content in EPS suggests less biofilm production during food grade exposures, other phenotypic parameters (relative hydrophobicity and EPM) exhibit similar trends between conditions indicating similar microbial community deposition and transport whether or not a TiO_2 exposure has occurred.

These findings further suggest minimal direct interaction between the particles is occurring, but rather that TiO_2 particles are interacting with molecules in the colon medium suggesting the development of the protein corona around the nanomaterials. Preferential interactions with proteins, and other molecules present within the medium would render the particles and molecules biologically unavailable to the microbial community. Lastly, these findings support the utilization of particles most likely present within the system of study to perform environmental fate, transport, and toxicity assessments. Within this system, food grade TiO_2 exposure has been shown to result in different microbial compositions and biochemical responses than both industrial grade exposure and baseline conditions indicating industrial grade may not accurately represent impacts of TiO_2 particles within the human body.

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Chapter 3

Influence of Food and Industrial Grade TiO_2 Nanoparticles on Microbial Diversity and Phenotypic Response in Model Septic System

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Chapter 3: Influence of Food and Industrial Grade TiO_2 Nanoparticles on Microbial Diversity and Phenotypic Response in Model Septic System

Abstract

This investigation focused on the affect that food grade titanium dioxide (TiO_2) nanoparticles, commonly found in consumer products, can have on the instrumental biological process of degrading household waste in septic systems. Microbial communities are instrumental in the sustainability of natural and engineered environments by degrading organic matter to products easily removed by other organisms. Wastewater treatment is one such engineered environment, where large scale facilities use activated sludge and other processes to reduce organic matter and waste reintroduced into water systems. TiO_2 nanoparticles represent a commonly detected contaminant in the wastewater influent reaching treatment facilities. However, while the influence of TiO_2 nanoparticles in large scale, multi-stage treatment operations has been well studied, decentralized septic systems, utilized in 25% of United States homes, are neglected. Unlike centralized water treatment facilities, septic system function is entirely dependent on the health of the microbial community. Thus, this study focused on the characterization of the septic system's microbial community and water quality in response when exposed to a common consumer product, food grade TiO_2 (FG), and an extensively researched nanoparticle, industrial (IG) grade TiO_2. Notably, FG and IG TiO_2 exposure resulted in distinct responses in select microbial and effluent quality parameters. Additionally, a more diverse microbial community composition developed during FG TiO_2 exposures, indicating that FG TiO_2 may alter microbial relationships affecting anaerobic digestion efficiency. Results indicate

that nano-FG TiO_2 may cause considerable changes to microbial function in septic systems and that understanding downstream effects requires studying ecologically relevant forms of nanoparticles.

3.1 Introduction

Engineered nanomaterials, such as titanium dioxide (TiO_2), are commonly incorporated into foods (Abbas *et al.* 2009; Bouwmeester *et al.* 2009), as well as pharmaceuticals and personal care products (Weir *et al.* 2012) to enhance the quality of consumer products. Multiple studies have determined engineered nanomaterials are routinely released from products during the normal lifecycle (Benn et al. 2008; Gottschalk et al. 2009; Mueller et al. 2009; Brar et al. 2010; Kim et al. 2010; Kaegi et al. 2011). TiO_2 nanomaterials released from consumer products, such as cosmetics, food coatings and, pigments, are present in detectable quantities (e.g. 0.3 mg/L TiO_2) in influent streams at wastewater treatment plants (WWTPs)(Westerhoff *et al.* 2011; Shi *et al.* 2016). Removal of nanomaterials in conventional WWTPs is found to be largely dependent on nanoparticle-biomass association prior to waste stream sedimentation (Westerhoff *et al.* 2011; Westerhoff *et al.* 2013). However, while micron sized TiO_2 aggregates were reported to be well removed (96.1-99.4% removal), over 70% of the nano-sized titanium passed through the WWTP (Kiser *et al.* 2009; Westerhoff *et al.* 2011).

Challenges associated with nanoparticle (NP) removal in conventional treatment systems make decentralized wastewater treatment applications (e.g. septic systems) an area of concern. A septic tank is an anaerobic digester driven by microbial degradation of organic compounds prior to release of waste stream into groundwater systems (Canter 1985). Insufficient maintenance and monitoring leads to septic system overloading and failure (EPA 2011; Taylor *et al.* 2015). Previous investigations of industrial grade forms of nano-

TiO_2 revealed potential cyto- and genotoxic effects to mammals (Jani *et al.* 1994; Trouiller *et al.* 2009) and aquatic organisms (Zhang *et al.* 2007) suggesting this nanomaterial is potentially detrimental to the microbial environment within the septic treatment system. Additionally, conventional WWTPs possess the added benefit of periodic biomass removal that extracts a fraction of nanoparticles, whereas septic systems are likely to accumulate bound nanoparticles due to longer biomass retention times (Canter 1985; Withers *et al.* 2014). Extended contact periods between nanoparticles and biomass, as well as with microbial communities influences bioavailability of nutrients for bacteria and subsequently, alters solution conditions of the biological system (Habimana *et al.* 2011; Peulen *et al.* 2011; Sahle-Demessie *et al.* 2011). Thus, nanoparticle presence becomes an additional hazard to this waste treatment method and downstream ecological processes.

The need to design experiments using environmentally relevant form of nanomaterials was recognized in that, although industrial grade (IG) TiO_2 has been more commonly used in environmental impact studies (Weir *et al.* 2012; Yang *et al.* 2014), food grade (FG) TiO_2 is more likely to be present in aquatic and wastewater environments (Weir *et al.* 2012). Previous investigations have demonstrated that environmentally relevant nanoparticle parameters such as elemental composition, crystal structure, size, and surface composition were observed to be notably different between the two TiO_2 types in low ionic strength solutions (Yang *et al.* 2014). Yang et al., (2014) determined primary particle sizes to be 100 % nano (<100 nm) for IG and 30 % for FG TiO_2. Differences in crystal structure were also observed in that IG TiO_2 was found to be comprised of a compound crystal structure of anatase (75 %) and rutile (25 %), while FG was determined to be solely anatase (>98 %). Additionally, a phosphate coating, presumably for stability, was associated with FG particles, and more readily adsorbed cationic dyes than IG, which lacked a coating of any sort (Yang *et al.* 2014).

Differences were also observed between food and industrial grade TiO_2 systemic effects in high ionic strength solutions containing sugars and proteins consistent with colonic waste as the model organic materials (Waller *et al.* 2017). FG exhibited a remarkably stable particle size (~300 nm) across changing pH (4-8) in both low and high ionic strength solutions (10 mM and 190 mM, respectively), while IG was far more susceptible to aggregation (e.g. ~900 nm at pH 6, 10 mM KCl). Microbial compositional differences were also noted as FG TiO_2 prevented a *Proteobacteria* to *Firmicutes* community shift observed during the control, whereas IG exposures merely slowed the process. These studies clarified that differences in environmentally relevant nanoparticle parameters, observed under ideal conditions, could also create an impact in more complex solutions. Whether the differences between the two types of TiO_2 nanoparticles could affect the wastewater microbial community to the extent to influence treatment efficiency (e.g. septic tank performance) remain to be investigated.

This study aimed to address this gap in knowledge. Specifically, an *in vitro,* lab scale model human colon provided a consistent material (both in microbial community and constituents of fecal matter) for subsequent introduction into a lab scale model septic system (Marcus *et al.* 2013; Taylor *et al.* 2015). Microbial phenotype, community structure, and effluent quality were indicators of system performance and perturbations from food and industrial grade TiO_2 nanoparticle exposure. Microbial phenotype focused on biofilm related parameters like hydrophobicity and extracellular polymeric substance production. Microbial community composition mapped changes to community structure via 16S rRNA sequencing and effluent quality monitored degradation of organic matter and system acidic buffering capacity. This study elucidates whether engineered modifications of the model nanomaterial (TiO_2) cause dissimilar behaviors under complex conditions found in wastewater systems.

3.2 Experimental Protocols

3.2.1 Experimental Design

Experiments were conducted using previously described bench scale model reactors of a human colon and septic tank (Marcus *et al.* 2013; Taylor *et al.* 2015; Waller *et al.* 2017). Fecal material introduced to the septic tank was produced using a model colon described in detail elsewhere (Marcus *et al.* 2013). Briefly, the model septic system is a scaled down model of an actual septic tank containing primary and secondary sedimentation chambers of 144 L and 72 L, respectively. Experimental runs comprised three consecutive weeks of a baseline control phase (no NP introduction), followed by a three-week exposure phase to the particular TiO_2 nanoparticle (FG or IG), and a three-week recovery phase (no NP introduction) to monitor the persistence of any perturbations resulting from nanoparticle exposure. A three-week period was selected for each of these phases as it mimics the retention time of effluent in an actual septic system (EPA 2002). The colon medium, containing simple salts, proteins, and carbohydrates, was fed through the colon reactor to produce the fecal matter used in these experiments (Marcus *et al.* 2013). During the exposure phase, TiO_2 nanoparticles were added to the colon medium at the start of the exposure phase to use “aged” rather than pristine TiO_2.

Fecal matter was collected from the model colon (100 mL) at equal intervals three times per day and immediately transferred into the model septic tank along with 71 mL of gray water (5x concentrated) and 5079 mL of Millipore water to simulate flushing. Gray water accounts for wastewater inputs from household sources other than fecal material containing, per liter, 20 mg humic acid, 50 mg kaolin, 50 mg cellulose, 0.5 mM $CaCl_2$, 10 mM NaCl, and 1mM $NaHCO_3$ per liter at pH 8 (Nghiem *et al.* 2006).

Twice weekly, samples for characterizing the microbial community and wastewater quality were collected from secondary effluent obtained from the base of the septic tank’s secondary chamber. Results presented are normalized by the average of the baseline values (recorded over three weeks) to indicate any measurable change from the initial control values. The control values are referred to as ‘Week 1’ throughout the remainder of the manuscript.

3.2.2 TiO_2 Particle Selection and Characterization

Food grade (FG) and industrial grade (IG) TiO_2 were utilized in this study. These nanoparticles were selected based on FG TiO_2 being the most likely type to reach wastewater treatment facilities from pharmaceuticals and personal care products, while IG is more commonly utilized in environmental fate and toxicity studies (Weir *et al.* 2012). FG particles are commercially available nanomaterials (provided by Arizona State University) and IG nanoparticles were acquired from Sigma Aldrich (Aeroxide TiO_2 P25; Evonik Degussa Corporation, Essen, DE). FG particles were reported to be 122 ± 48 nm in primary size, having a > 95% anatase crystal structure, and also with an inorganic phosphate coating (Yang *et al.* 2014). IG nanoparticles have been well characterized previously with a primary particle size of 21 nm, a compound crystals structure of 75% anatase to 25% rutile, and no presence of an engineered surface coating (Yang *et al.* 2014; Waller *et al.* 2017). TiO_2 exposure concentration (36 mg TiO_2 per day) was based on average daily consumption estimates for an adult of 0.45 mg per kg of body weight (Weir *et al.* 2012) assuming an 80 kg adult male (Walpole *et al.* 2012).

3.2.3 Phenotypic Characterization

Microbial community characterization was performed on bacteria extracted from the secondary chamber septic tank effluent, representing what would otherwise be introduced into a leach field and groundwater system in an actual system. Cell suspensions that were collected were both washed in triplicate and suspended in 10 mM KCl using a centrifuge (Eppendorf 5804R, Hamburg, DE) operated at 3,700 *x g* for 15 minutes. Electrophoretic mobility (EPM) was quantified to approximate the relative surface potential (ZetaPALS, Holtsville, NY, USA) of the microbial community. Relative hydrophobicity was determined by the microbial adhesion to hydrocarbon test (MATH) using n-dodecane as the model hydrocarbon. Relative hydrophobicity uses partitioning to quantify the relative presence of nonpolar functional groups at the microbial surface. Microbial extracellular polymeric substances were quantified using the freeze-dry method and the bound sugar and protein (s/p) ratios were colorimetrically determined (sugar: 480 nm; protein: 500 nm) with a UV-vis spectrophotometer (Shimadzu Bio-mini, Kyoto, JPN) (Gong *et al.* 2009). Microbial characterization methods are described in more detail in previous work (Waller *et al.* 2017) .

3.2.4 Microbial Community Sequencing

Microbial community DNA sequencing allowed the characterization of the baseline structure and any measurable changes to the septic system microbial community resulting from the introduction of the nano-TiO_2 particles. On the first day of each experimental week, 240 mL samples of secondary chamber effluent were centrifuged at 3,700 *x g* and resuspended in 4 mL of 10 mM KCl. Subsequently, microbial cells were sampled from 1 mL aliquots of the secondary effluent and the DNA was extracted using the MoBio PowerFecal® DNA Isolation Kit (Carlsbad, CA, USA) according to manufacturer protocols. Extracted DNA was stored in a -80°C freezer until they were shipped to Research and Testing Laboratories (Lubbock, TX, USA) for 16S rRNA sequencing. Universal primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used to obtain data regarding bacteria and archaea compositions.

3.2.5 Septic System Function

Monitoring septic system function was achieved by measuring specific water quality parameters (Rice *et al.* 2012). Water quality parameters tested included alkalinity, pH, total dissolved solids (TDS), as well as representative volatile fatty acids (VFAs); these tests were performed in accordance with standard methods (Rice *et al.* 2012). Samples were tested immediately after being collected from the secondary septic tank chamber effluent twice weekly. Electronic probes were used for determining TDS (YSI 3200 Conductivity Instrument Model #3200 115 V, YSI, Yellow Springs, OH, USA) and pH (Thermo Scientific Orion Star A214, Waltham, MA, USA). Quantification of representative VFA (acetic, propionic, and butyric acids) concentration utilized a previously published method (Venema *et al.* 2003; Taylor *et al.* 2015). Briefly, samples were centrifuged at 12,000 x rpm for 5 min before a 50 μL effluent aliquot was mixed with 650 mL internal standard, by mass ratio, 20 g of formic acid, 80 g of Millipore water, 434 g of methanol, and 200 mg 2-ethyl butyric acid. VFA concentrations were calculated using GC-FID (Agilent, Santa Clara, CA, USA) via peak integration under flame ionization curve and fitted to the respective calibration curves.

3.2.6 Statistical Analysis

Statistical significance was determined between the baseline conditions (control week 1) and each individual exposure week (2, 3, or 4), as well as each individual recovery week (5, 6, or 7) by student t-test. An alpha value of 0.05 (p-value < 0.05) indicated statistically significant differences resulted from the introduction of FG or IG TiO_2 nanoparticles.

Additionally, three separate correlative analyses were performed to identify potentially less obvious, but significant trends ($p\text{-value} < 0.01$) between weekly microbial compositional trends for (1) IG exposure, (2) FG exposure, and (3) all phenotypic parameters for both FG and IG exposures combined.

3.3 Results

3.3.1 Septic System Function

Parameters measured as indicators of effluent quality are presented in Figures 3.1-4; error bars indicate standard deviation of triplicate measurements. Baseline conditions comprise data points from the 3 weeks of baseline averaged to reflect a standard week to simplify the analysis of TiO_2 exposure. Exposure and recovery weeks are normalized by the baseline values. Total dissolved solids (TDS) are a measurement that could be used to quantify the ionic content of the effluent providing an indicator of the fate and transport of wastewater compounds. FG introduction essentially had no effect on TDS (Figure 3.1C). Conversely, TDS declined greatly during IG introduction, falling progressively each week to 49.2 ± 2.0 % of the initial value by the end of the exposure phase (Figure 3.1C). TDS rose for both TiO_2 exposure conditions during the recovery phase as a delayed response with FG reached 16.7 ± 11.0 % above baseline conditions while IG only recovered to 66.7 ± 0.0 % of its initial levels. IG exposures result in a significant pulse-like, possibly persistent, decrease of effluent TDS, while FG appears to have little effect during the exposure phase.

Alkalinity measures the septic systems capacity to resist changes in pH from acid produced during anaerobic, microbial metabolism and it provides an indicator of the system's acidic buffering capacity. Neither TiO_2 exposure caused major changes in pH, meaning acid presence never exceeded the systems buffering capacity (FG: 4.0 ± 3.0 % rise; IG: 4.3 ± 1.0 % decrease) (Figure 3.1A). Inversely related system alkalinity trends were observed between FG exposure which resulted in 10.8 ± 5.0 % increase and IG exposure which caused a 19.5 ± 3.0 % decrease (Figure 3.1B). Alkalinity in the FG recovery phase increased an additional 36.5 ± 15.0 % above initial showing persistent effects as IG recovery phase returned within 4.8 ± 8.0 % of its initial value. These results demonstrate that IG exposures result in a non-persistent increase in alkalinity consumption, associated with greater acid content, whereas FG appears to have little effect.

Volatile fatty acids are intermediate products of anaerobic digestion related to the mineralization of organic compounds. Interestingly, propionic and butyric acid did not deviate from initial, baseline conditions during both FG and IG TiO_2 exposures (Figure 3.2). Acetic acid, however, drops to a minimum of 66.7 ± 13 % of initial conditions before ending the FG exposure phase at 16.7 ± 30.0 % of the baseline value (Figure 3.2B). The inverse is observed with IG exposures resulting in a 30.3 ± 30.0 % increase above the initial conditions before plunging 74.3 ± 13.0% of the baseline conditions by the end of the exposure phase (Figure 3.2A). Acetic acid levels of both TiO_2 exposure scenarios remain below initial conditions during the recovery phase never fully returning to baseline values. IG exposures may serve to stimulate acetic acid production while FG may stall production, although both systems appear to reach a new equilibrium in the recovery phase below the initial system conditions.

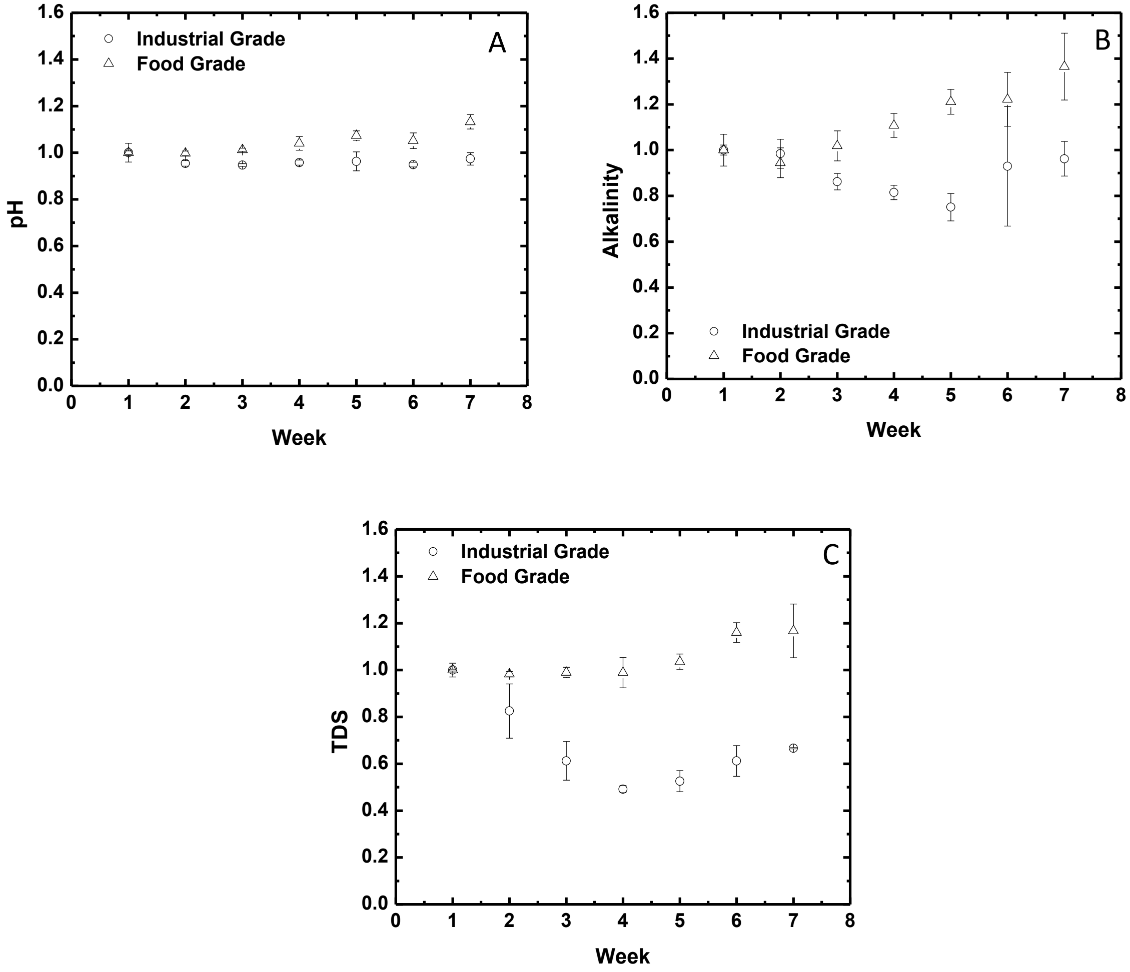

Figure 3.1: Weekly averages of (a) pH, (b) alkalinity, and (c) total dissolved solids for both food and industrial grade TiO_2 normalized to initial system conditions. Week 1 represents the initial baseline (control), TiO_2 particles are introduced into the system during weeks 2-4, and weeks 5-7 are the recovery phase when TiO_2 particles are no longer introduced in the system Error bars represent triplicate measurements of standard deviation.

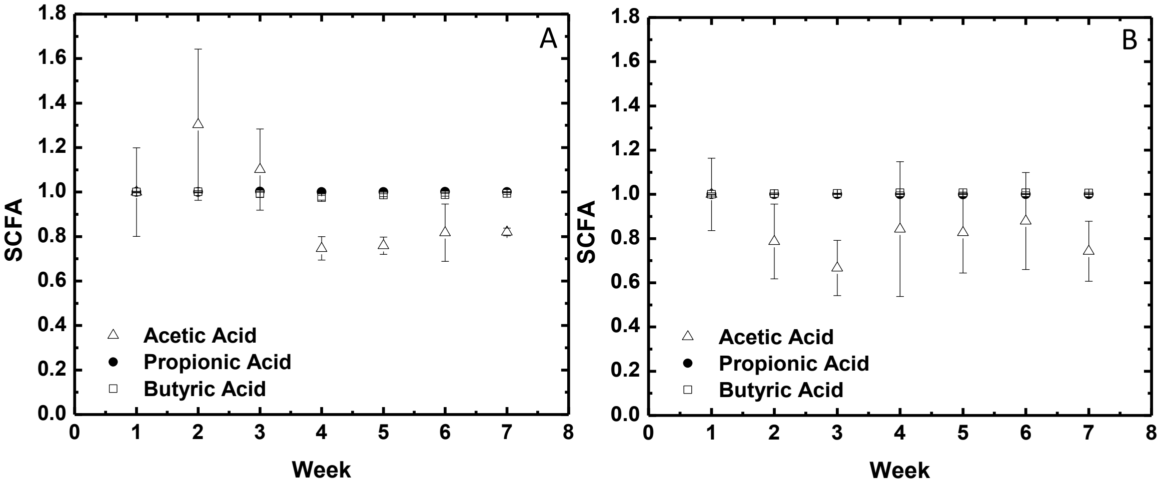

Figure 3.2: Normalized weekly averages of short chain fatty acids (SCFA) for (a) industrial grade and (b) food grade TiO_2 normalized to initial system conditions. Week 1 represents the initial baseline (control), TiO_2 particles are introduced into the system during weeks 2-4, and weeks 5-7 are the recovery phase when TiO_2 particles are no longer introduced in the system. Error bars represent triplicate measurements of standard deviation.

3.3.2 Microbial community composition

The microbial community composition was determined using pyrosequencing and indicates relative abundance based on reads (Figure 3.3). Initial baseline conditions for each separate run were similar at the phyla level (*Proteobacteria* dominant, FG: 83% and IG: 75%), diverging as the taxa level decreased towards genera. Dominant genera comprising the *Proteobacteria* phylum are: *Acineotobacter, Comamonas, Enterobacter, Azospirillum, Azospira, and Pseudomonas. Clostridium and Bacteroidetes* are the dominant genera from the Firmicutes and *Bacteroides* phyla, respectively. Relative abundance of each genera account for 80% and 79% of the initial sequenced microbial community for FG and IG runs, respectively. The genus of the FG's initial microbial population was composed of *Acineotobacter* (47.6%)*, Clostridium* (12.6%)*, Comamonas* (10.5%)*, Enterobacter* (8.4%)*, and Azospirillum* (4.4%) (Figure 3.3B). IG's initial microbial population comprised of *Azospira* (31%)*, Azospirillum* (28.6%)*, Bacteroides* (8.4%)*, Comamonas* (5.6%), and *Pseudomonas* (5.2%) (Figure 3.3A).

The introduction of FG particles to the microbial community quickly caused *Acinetobacter* to decrease 13.9 % (33.7 % relative abundance) during the first week of exposure prior to falling further to 17.9 % relative abundance during the recovery phase (Figure 3.3B). *Enterobacter* and *Comamonas*, conversely, became more prevalent during exposure phase increasing 5.1 % (13.6 % relative abundance) and 2.8 % (13.3 % relative abundance), respectively before *Comamonas* increased further to 20.5 % relative abundance in the recovery phase. IG introduction had little effect on the relative abundance of the *Azospira* and *Azospirillum* dominant community resulting in a 3 % increase (34 % relative abundance) and 0.7 % decrease (27.9 % relative abundance), respectively, at the end of IG exposure phase (Figure 3.3A). Inversely related trends developed as *Azospira* was then reduced 13.4 % (20.6 % relative abundance) during the recovery phase while *Azospirillum* increased 15.8 % (43.7 % relative abundance). IG exposure conditions were more conducive to the nitrogen fixing genera of *Azospira* and *Azospirillum* while FG presence was detrimental to the relative quantity of *Acinetobacter*.

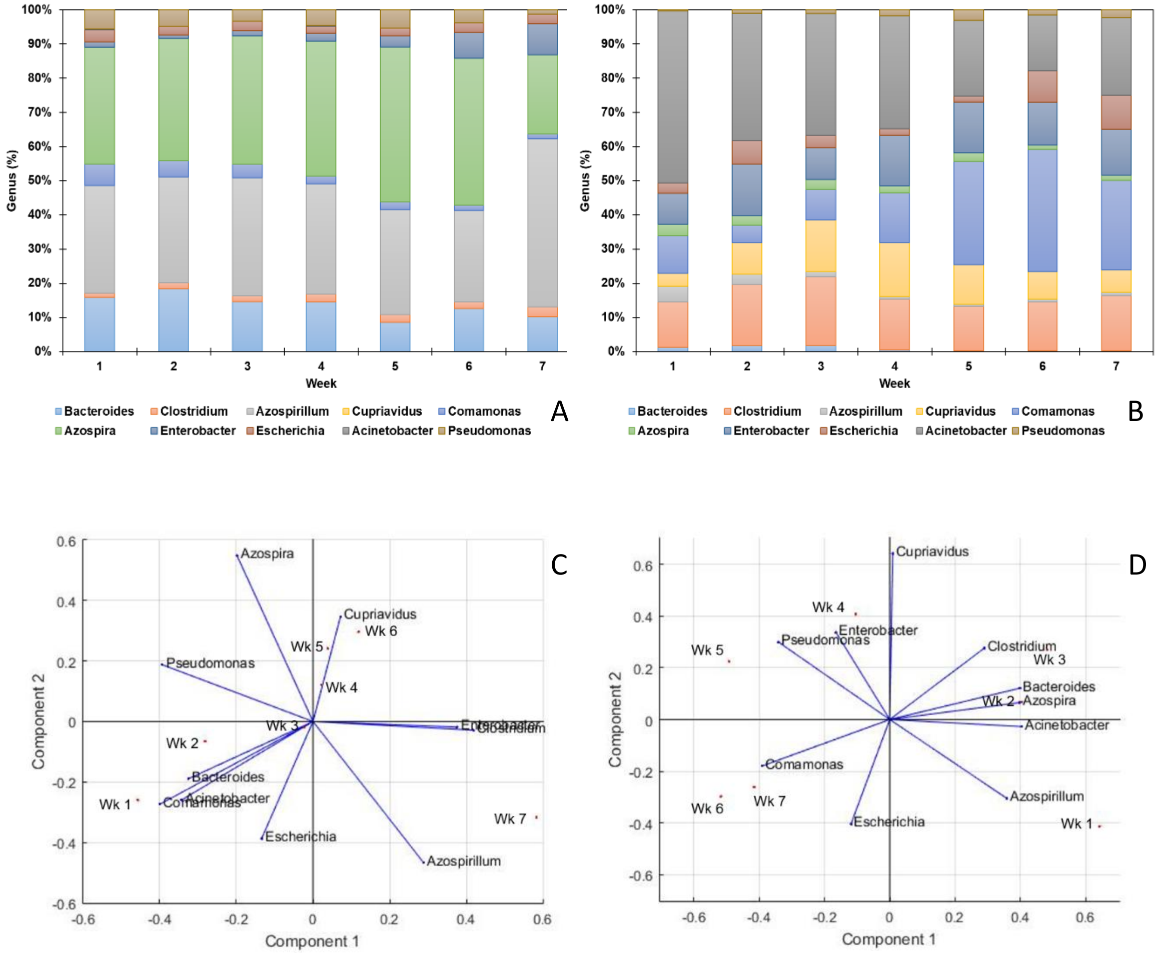

Figure 3.3: Microbial community fingerprint (IG: A, FG: B) and principle component analysis of microbial Genus taxa rank (IG: C, FG: D). Week 1 represents the initial baseline (control), TiO_2 particles are introduced into the system during weeks 2-4, and weeks 5-7 are the recovery phase when TiO_2 particles are no longer introduced in the system.

3.3.3 Phenotypic characterization

Electrophoretic mobility (EPM) quantifies the stability of the microbial community based on interactions between the microbial surface and solution conditions. Increases in EPM reflect a more stable and therefore more mobile microbial community (Marcus et al., 2013), which also is associated with reduced biofilm presence. The FG exposure phase resulted in a 15.1 ± 11.0 % increase of microbial community EPM (Figure 3.4A). The increased mobility is persistent as EPM remained 9.2 ± 3.0 % above the initial, baseline conditions. Microbial EPM increased in the first two weeks of IG TiO_2 exposures, although with large variability 8.0 ± 8.0 %, prior to initial conditions redeveloping by the end of the recovery phase (Figure 3.4A). While both communities exposed to TiO_2 particles exhibit greater mobility, the IG exposed community recovers to initial conditions as the FG exposed community has more persistent effects and suggest a decreased biofilm presence.

Relative hydrophobicity provides insight into the presence of nonpolar molecules at the microbial surface; a process usually associated with the development of biofilm (Marcus *et al.* 2012). FG exposure resulted in a decrease to 83.0 ± 17 % of initial relative hydrophobicity before rising to 28.1 ± 10 % by the end of the recovery phase (Figure 4B). Conversely, microbial hydrophobicity increased 61.6 ± 8.6 % during the IG exposure phase (Figure 3.4B). The increased hydrophobicity persisted through the recovery phase with final values of 1.7 ± 0.7 %, although large variability was observed. Greater biofilm production is expected from the IG exposed community than FG based on the increased and sustained hydrophobicity.

Sugar to protein ratio (s/p ratio) compares the sugar and protein content of extracellular polymeric substances (EPS) as an approximation of biofilm production where an increased s/p ratio indicates greater tendency for biofilm development (Marcotte et al., 2007). Microbial community exposure to both IG and FG TiO_2 nanoparticles caused increased s/p ratios of 9.0 ± 11 % and 29.8 ± 11.0 %, respectively, above the initial conditions (Figure 4C). Signs of recovery were evident during IG recovery phase as values decreased to 87.8 ± 17.0 % of the initial conditions whereas the s/p ratio during FG recovery remained 9.9 ± 10.0 % above the initial (Figure 3.4C). Findings suggest both TiO_2 nanoparticle exposed communities exhibit biofilm forming trends with FG exposure effects more persistent. The IG exposed community reflects an adaptive and robust nature indicating an ability to withstand changing system conditions.

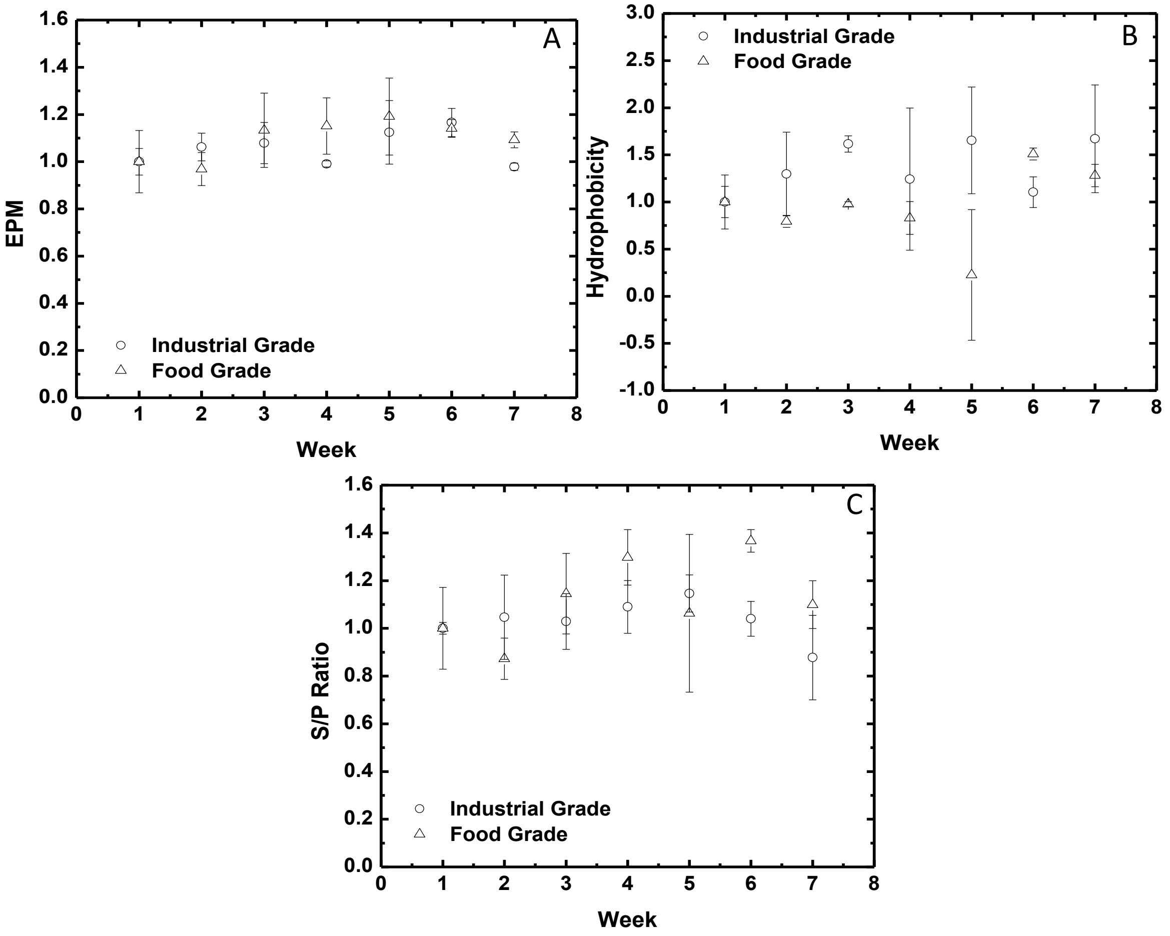

Figure 3.4: Weekly averages of (a) hydrophobicity and (b) electrophoretic mobility for both food grade and industrial grade TiO_2 normalized to initial system conditions. Sugar to protein (S/P) ratio (c) is presented as non-normalized weekly averages. Week 1 represents the initial baseline (control), TiO_2 particles are introduced into the system during weeks 2-4, and weeks 5-7 are the recovery phase when TiO_2 particles are no longer introduced in the system Error bars represent triplicate measurements of standard deviation.

3.4 Discussion

3.4.1 Septic System Function

Previous research has suggested that the risk of anthropogenic environmental damage from emerging contaminants is minimized when waste degradation remains optimal and the septic system remains balanced (Canter 1985). Septic systems are microbially driven anaerobic digesters that are failure prone due to both acute and chronic contaminant loadings (EPA 2002). Monitoring key septic system metrics like VFA production, alkalinity, pH, and TDS aid in understanding system performance by quantifying acid production, system acid buffering capacity, and ionic strength of the treated effluent (Rice *et al.* 2012) . In this study, notable differences in septic tank effluent quality trends suggests that FG and IG TiO_2 exposure each had a unique effect on septic system function.

VFAs —acetic, propionic, and butyric acids — are intermediate products of anaerobic digestion that indicate the mineralization efficacy of organic wastewater compounds. Acetic acid, notably, accounts for up to half of the VFAs introduced into the waste system (Rios-Covian *et al.* 2016). Interestingly, IG and FG exposures caused inverse trends in acetic acid levels in the first two weeks of the exposure phase (Figure 3.2). Acetic acid levels immediately decreased to 66.7 ± 13 % of the initial level during FG exposures, while rising 30.3 ± 30 % during the initial IG exposure. Acetate is an essential substrate in methane production from anaerobic digestion (Blaut 1994; Bitton 2005) meaning its variation between TiO_2 exposures reflects distinct influences to the complete mineralization of organic matter. Additionally, it was observed that neither TiO_2 exposure had a significant effect on butyric and propionic acid levels. Acetate presence has the capacity to hinder the degradation of propionic and butyric acids (Kaspar *et al.* 1978; Ahring *et al.* 1988) potentially reflecting a more significant mechanism than solely TiO_2 presence governing the conversion of these two fatty acids. It is inferred that the initial IG introduction may boost mineralization by increasing acetic acid levels, although ultimately both exposures result in reduced quantities and the potential for hindered septic system function over the long term.

IG and FG TiO_2 exposures also resulted in dissimilar responses to septic systems acidic buffering capacity. Sudden or extreme changes in system pH can disrupt anaerobic digestion by inhibiting pH sensitive microbes (e.g. methanogens) highlighting the need for conditions to remain balanced and consistent (Bitton 2005; Taylor *et al.* 2015). Alkalinity consumption by acid production as pH remained constant during IG exposures demonstrated increased effluent acidity; although the levels did not exceed the system limits (Figure 3.1A-B). Acetic acid increases observed during the IG exposure phase support the observation that acidity was indeed greater than initial conditions (Figure 3.2A). The septic system exposed to IG TiO_2 demonstrates continued operation with increased acidity, yet a resilient, robust nature as system effects were non-persistent. Interestingly, however, rising alkalinity accompanying a slight pH increase during FG exposures, continuing into the recovery phase, reflects an alternate response than during the IG run. An unexpected decrease in acetic acid production (Figure 3.2B), indicates FG exposures may inhibit earlier stages of anaerobic digestion such as hydrolysis, acidogenesis, and acetogenesis (Bitton 2005). It is well understood that nanoparticles in biological systems develop organic coatings and associate with biomass (Cedervall et al. 2007; Lynch et al. 2008; Kiser et al. 2010; Tong et al. 2010; Westerhoff et al. 2011; Westerhoff et al. 2013; Ikuma et al. 2015), which allows the possibility for reducing bioavailability of anaerobic bacteria substrates.

Major differences in the quantity of total dissolved solids reveal ionic strength as another effluent quality parameter that varied by which form of TiO_2 the septic system was exposed to. TDS values plunged in the first week (week 2) of IG grade exposures before rising again during the recovery phase (Figure 3.4C). Lower TDS reflects improved water quality and results from biological activity such as ion uptake by the microbial community (Bian *et al.* 2011), as well as, sedimentation from wastewater flocs (Bian *et al.* 2011; Crittenden *et al.* 2012). However, wastewater biomass and bacteria that remain in suspension would possess greater stability and are likely to travel further upon release from the system (Zita *et al.* 1994; Chowdhury *et al.* 2012). FG TiO_2 presence, conversely, caused little deviation in TDS and even increased above initial values during system recovery. IG TiO_2 has been shown to have much larger aggregate sizes compared to FG in both simple and complex media (Waller *et al.* 2017) and therefore may provide a binding surface for charged biomass to form colloids with greater probability of settling out of suspension (Westerhoff *et al.* 2011; Westerhoff *et al.* 2013). Effectively, it can be stated that improved water quality resulted from IG exposure, although suspended particles may be stabilized, while FG had negligible or detrimental effect on system TDS and effluent quality as a result.

3.4.2 Septic Effluent Microbial Community Composition

Wastewater microbial communities drive biological treatment processes by efficiently degrading organic compounds in raw sewage (Ye *et al.* 2013). Microbial communities are complex compositions maintaining symbiotic relationships that influence factors associated with process control and system optimization (Cydzik-Kwiatkowska *et al.* 2016). Figure 3.3A and B display a graphical representation of microbial community genera percentage from the baseline community (Week 1) for IG and FG exposure runs through exposure and recovery, respectively. At the phylum level, the microbial communities are quite similar with both being *Proteobacteria* dominant (FG: 83% and IG: 75%). This microbial composition agrees with prior studies of septic systems (Tomaras *et al.* 2009; Marcus *et al.* 2013; Taylor *et al.* 2015), as well as, communities present in full scale wastewater treatment samples (Ye *et al.* 2013; Cydzik-Kwiatkowska *et al.* 2016). However, composition at the genera taxa rank show differences in the initial community as IG is comprised of largely *Azospira* (31%) and *Azospirillum* (28%) while FG is dominated by *Acinetobacter* (50%). This difference in community composition may result from biofilm growth as microbes existing within an EPS matrix experience a complex "micro-niche" facilitating homeostasis and a nutrient circulatory system (Costerton *et al.* 1995), the balance of which influences the genera that thrive. Additionally, factors like competition, microbial dispersal from growth and death, or mixing as influent enters the system can further cause compositional heterogeneity (Cydzik-Kwiatkowska *et al.* 2016).

TiO_2 exposure to these respective baseline communities caused notably different responses to microbial community compositions. IG TiO_2 introduction (Figure 3.1A Week's 2-4) effectively caused no change on the denitrifiers *Azospira* and *Azospirillum*. Denitrifiers play a critical role in completing the mineralization of organic matter in biological treatment systems (Chen *et al.* 2014) making their presence ideal for optimum wastewater treatment. *Azospira* and *Azospirillum* persistence indicates IG TiO_2 exposure may facilitate denitrifier activity. Partial support for this is seen with surges in acetic acid observed in the first two weeks of particle introduction, reflecting an increased presence of nutrients while decreases in TDS suggests mineralization of VFAs existing as organic anions (Nugent *et al.* 2001).

FG TiO_2 exposure*,* conversely, considerably affected *Acinetobacter* (decreasing 16 % to comprise 30 % of community) simultaneously as *Enterobacter* and *Comamonas* gained larger footholds in the community (14 % and 13 %, respectively). *Comamonas* increased further (7 %) in the recovery phase, perhaps better able to persist with a less dominant *Acinetobacter* presence. *Comamonas* represents one of many heterotrophic nitrifying genera capable of consuming ammonia, although autotrophs may be more ideal under anaerobic conditions (Cydzik-Kwiatkowska *et al.* 2016). Symbiotic relationships that develop between bacteria ideally provide the microbial communities' robust qualities, making them resilient under dynamic conditions (Arumugam *et al.* 2011); however, FG TiO_2 presence appears detrimental to the adaptive nature of the microbial community. Water quality findings of the current study support that the wastewater treatment efficiency is impaired (Figures 3.1 and 3.2) during FG exposures. Interestingly, *Acinetobacter* has been shown to exhibit antibiotic resistance and the ability to metabolize acetate in anaerobic wastewater (Kim *et al.* 1997) indicating FG TiO_2 may interfere with factors aiding *Acinetobacter* resilience and this particular metabolic pathway. Ultimately, while IG had little effect on a primarily denitrifying dominant community, FG exposure enabled a shift to a more diverse community that exhibited less than ideal functionality for wastewater treatment.

3.4.3 Phenotypic Characterization

Exposure of the wastewater microbial community to FG and IG TiO_2 resulted in changes to the microbial phenotype capable of influencing biofilm production. Quantifying the ratio of sugar and protein (s/p) in the EPS in this study aided the prediction of the possibility of biofilm formation (Eboigbodin *et al.* 2008), while microbial hydrophobicity and EPM served as qualitative indicators of biofilm development around the microbial cell surface (Schafer *et al.* 1998; Marcus *et al.* 2013; Waller *et al.* 2017). Inversely related trends of biofilm formation are expected to develop between FG and IG exposed microbial communities based on microbial hydrophobicity, EPM, and s/p ratios, with elevated levels expected from the IG exposed community (Figure 3.4). Interestingly, although the FG exposed microbial community became more hydrophilic during exposure, persistent hydrophobic trends developed for both communities during the recovery phases. Hydrophilic bacteria have a tendency to exist in a planktonic state (Costerton *et al.* 1995;

Flemming *et al.* 2010) indicating a more mobile microbial community developed during FG exposure conditions (Figure 3.4A).

Delayed surges in hydrophobicity may indicate microbial community acclimation to starvation conditions brought on by FG TiO_2 nanoparticle exposure, where a dense biofilm may have been detrimental to community survival (Flemming *et al.* 2010). Increases in microbial biofilm production from IG TiO_2 nanoparticle exposures may prove beneficial to the microbial community as a filter network trapping organic molecules and essential ions while, ideally, excluding deleterious substances (Ikuma *et al.* 2015). Additionally, increased biomass may also be beneficial to water quality as greater surface area is available for adsorption and sedimentation of organic (Cedervall *et al.* 2007; Kiser *et al.* 2010; Ikuma *et al.* 2015) and inorganic compounds (Westerhoff *et al.* 2011; Westerhoff *et al.* 2013) , including IG TiO_2 nanoparticles, ultimately leading to nanoparticle removal from effluent streams (Cedervall *et al.* 2007; Lynch *et al.* 2008; Kiser *et al.* 2010; Tong *et al.* 2010; Westerhoff *et al.* 2011; Westerhoff *et al.* 2013; Ikuma *et al.* 2015).Whereas biomass produced in full scale treatment operations is periodically removed, the biomass – and nanoparticles (Taylor *et al.* 2015)– accumulates in the sedimentation chamber of a septic system making long-term exposure studies an area deserving further research.

3.4.4 Principle component and correlation analyses

Septic system response to the exposures of FG and IG TiO_2 were elucidated statistically using principle component (PCA) and correlation analyses. Observations presented represent correlations between testing parameters resulting from systematic changes and not necessarily “cause and effect” relationships. PCA describing weekly variations in relative abundance of the microbial community genera are presented for IG and FG exposure runs in Figures 3.3C and D, respectively.

Both types of TiO_2 showed substantial impact on the microbial community within the septic tank as presented above in the results, and the significance of this impact can be evaluated using principle component analysis (PCA). As principle component (PC) 1 reflects the component containing the majority of variation, it can be said that TiO_2 exposure resulted in persistent changes in the dynamics of the microbial community (Figure 3.3C and D). Relative microbial abundance shifts at the onset of IG exposure along principle component (PC) 1 (60 % of variation) reflect changes in the composition of the less abundant genera of the system (Figure 3.3C). Although the dominant members shown in Figure 3C (*Azospira* and *Azospirillum)* maintain a system balance and function, variations in less dominant genera are observed during the IG exposure, indicating the NP presence does influence community structure. The large variation observed between Weeks 1 and 7 along PC 1 reflects that the final community varied considerably from the original population. This suggests the establishment of a new “normal” in the community after IG TiO_2 exposure, potentially with different dynamics and roles in the anaerobic system.

Persistent changes in community dynamics from FG TiO_2 exposure was elucidated by variance along PC 1 (58 % of the variation). Relative abundance of *Acinetobacter* was strongly linked with variance with respect to PC 1 between initial conditions and the FG TiO_2 exposure and recovery phases (Figure 3.3B and D). Interestingly, the less abundant genera remained stable in the presence of FG TiO_2 before an inverse trend developed in the third exposure week (Week 4) (Figure 3.3D). As such, a considerable shift in the final community structure is supported by the minimal variance along PC 1 in FG recovery weeks (5, 6, and 7) in agreement with trends shown in Figure 3.3B. Water quality findings substantiate that changes in the symbiotic relationships naturally present and required for effective WWT are not disrupted during IG exposures, whereas waste treatment capacity is impaired after FG exposure.

Septic system response to TiO_2 exposure was further evaluated using the statistical correlations between weeks using microbial composition, water quality and phenotypic parameters (Table 3.1). In addition, correlations between all testing parameters were also evaluated (Table 3.2). Weekly correlation data supports water quality and phenotypic findings that septic system exposure to IG TiO_2 alters system conditions based on strong correlations observed between the initial conditions (Week 1) and the first IG exposure week ($r = 0.9$, $p < 0.01$) before correlation is lost in the remaining exposure weeks (Table 3.1). This reflects that systematic changes (i.e. introduction of IG TiO_2) affect weeks 1 and 2 proportionally; whereas, inversely related trends had developed during the recovery phase (Week 7, $r = -0.59$, $p < 0.01$) that indicate statistically significant conditions in the septic system developed after IG TiO_2 exposure. Weekly correlation data reflects that FG TiO_2 exposure resulted in statistically significant systemic changes in microbial genera and phenotypic trends as observed by negative correlations ($r = -0.59$, $p < 0.01$) between initial conditions and the FG recovery phase (Weeks 5, 6, and 7). Essentially, FG TiO_2 exposure facilitated changes in septic system conditions capable of diminishing treatment capacity as determined by water quality findings.

A

Week	1	2	3	4	5	6	7
1	-						
2	0.87*	-					
3	-0.01	0.33	-				
4	0.15	0.44*	0.671*	-			
5	0.12	0.31	0.53*	0.86*	-		
6	-0.59*	-0.75*	-0.63*	-0.79*	-0.73*	-	
7	-0.33	-0.31	0.17	0.23	0.30	-0.28	-

B

Week	1	2	3	4	5	6	7
1	-						
2	0.61*	-					
3	0.19	0.60*	-				
4	-0.51	-0.44	0.09	-			
5	-0.59*	-0.71*	-0.42	0.56*	-		
6	-0.56*	-0.66*	-0.63*	-0.14	0.16	-	
7	-0.56*	-0.60*	-0.73*	-0.15	0.23	0.88*	-
Note: * indicates $p < 0.01$							

Table 3.1: Correlation analysis of all phenotypic and genotypic parameters (weekly averages) for (a) industrial and (b) food grade TiO_2 exposure, respectively. Week 1 represents the control, weeks 2-4 were for TiO_2 nanoparticle exposure, and weeks 5-7 ceased TiO_2 nanoparticle exposure (recovery).

Parameter	Hydrophobicity	EPM	S/P Ratio	pH	Alkalinity	Turbidity	TSS	Ammonium	TDS	Acetic Acid
Hydrophobicity	-									
EPM	-0.39	-								
S/P Ratio	0.63	-0.92	-							
pH	0.31	-0.94	0.81*	-						
Alkalinity	-0.72	0.71	-0.83*	-0.59*	-					
Turbidity	-0.60	0.62	-0.71*	-0.48*	0.81*	-				
TSS	-0.65	0.73	-0.84*	-0.63*	0.961*	0.87*	-			
Ammonium	0.71	-0.80	0.86	0.77	-0.89	-0.67	-0.82	-		
TDS	-0.60	0.52	-0.55	-0.58*	0.43*	0.18*	0.30*	-0.77	-	
Acetic Acid	0.52	-0.80	0.83	0.71*	-0.87*	-0.72	-0.91*	0.81	-0.36	-
Note: * indicates $p < 0.01$										

Table 3.2: Correlation analysis of weekly averages of all phenotypic parameters combined for both industrial and food grade TiO_2 exposures.

Correlation analysis of water quality and phenotypic parameters provides useful analysis of system operation and function. Parameters were combined from both FG and IG TiO_2 exposures to highlight strong relationships (i.e. pH and alkalinity) that existed between both NP exposure scenarios to substantiate the accuracy of findings and system operation. Analyzing all of the data for NP exposure (both, FG and IG) can indicate that exposure to nanoparticles creates substantial system changes. Strong correlation between turbidity and TSS ($r = 0.87$, $p < 0.01$) aligns with relationships well known in literature (Crittenden *et al.* 2012) helping to validate the correlation analysis and supporting systems were operating properly. Notably, a strong positive correlation was also observed between pH and acetic acid ($r = 0.71$, $p < 0.01$), while strongly negative correlations were observed between alkalinity and acetic acid ($r = -0.87$, $p < 0.01$), as well as pH and alkalinity ($r = -0.59$, $p < 0.01$). Intuition suggests opposite trends would exist between pH and acetic acid concentration; however, the proportionality between the two is clarified by the inverse relationships demonstrated with acetic acid and alkalinity, as well as pH and alkalinity (Crittenden *et al.* 2012). These correlations reflect the dynamic nature of the system and emphasizes the significance of alkalinity in anaerobic digestion, as it allows chemical reactions to occur while maintaining system pH. Although FG and IG TiO_2 each influence microbial composition, phenotypic, and water quality parameters, consistent responses to the perturbation and the capacity of the system to recover highlight the robust nature of the microbial community and septic systems in general.

3.4.5 Summary

Historically, failing septic systems have been detrimental to groundwater quality when excessive nutrient loading and contaminants have been introduced into the system. Monitoring the effects of emerging contaminants (e.g. engineered nanomaterials) in decentralized systems is just as relevant as effects in centralized wastewater treatment plants as both have immediate environmental implications. As waste degradation in septic systems is an anaerobic, microbially driven process, potential impacts from nanomaterials to the microbial community must be characterized to understand potential causes of failure and downstream ecological effects from food grade TiO_2 nanoparticle presence.

Notable differences were observed in microbial phenotype and genera composition, as well as, septic system function from the introduction of food (FG) and industrial grade (IG) TiO_2 to the wastewater treatment system. Acute exposure of the septic microbial community to FG and IG TiO_2 particles ultimately resulted in inversely related tendencies for biofilm development. IG exposure lead to increased production of biofilm, while FG exposure lead to reduced biofilm production. Additionally, exposure of the septic system to IG TiO_2 particles had minimal effect on the composition of the denitrifying-dominant microbial community, while the FG exposures shifted the community structure to genera of mixed functionality, which is less effective at wastewater treatment. Lastly, septic effluent water quality may benefit in the short term from IG TiO_2 exposures by facilitating

VFA production, whereas exposure to FG nanoparticles may be detrimental to anaerobic digestion by interference in organic matter mineralization.

Valuable insight has been gained from this study concerning a need to focus on monitoring and controlling of nanomaterials used in foods and consumer products. It has been shown the wastewater microbial communities in a representative septic system is affected by food grade TiO_2 nanomaterial presence, although more research is needed to further assess the permeation through the groundwater post waste treatment. While nanomaterials may not directly affect human health, there exists the risk of unexpected ecological impacts occurring downstream of the initial consumer applications.

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Chapter 4

Influence of Septic System Wastewater Treatment on Titanium Dioxide Nanoparticle Subsurface Transport Mechanisms

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Chapter 4: Influence of Septic System Wastewater Treatment on Titanium Dioxide Nanoparticle Subsurface Transport Mechanisms

Abstract

Engineered nanomaterials (ENMs) are commonly incorporated into food and consumer applications to enhance a specific product aspect (i.e. optical properties). Life cycle analyses revealed ENMs can release from products during usage and reach wastewater treatment facilities (WWTPs), with titanium dioxide (TiO_2) accounting for a large fraction. As such, food grade (FG) TiO_2, a more common form of TiO_2 in wastewater, was used in this study. Nanomaterials in WWTPs have been well characterized, although the problematic septic system has been neglected. Elution and bioaccumulation of TiO_2 ENMs from WTTPs in downriver sediments and microorganisms has been observed, however little is known about mechanisms governing the elution of FG TiO_2 from the septic drainage system. This study characterized the transport behavior and mechanisms of FG TiO_2 particles in porous media conditions after septic waste treatment. FG and industrial grade TiO_2 (more commonly studied) were introduced to septic tank effluent and low ionic strength electrolyte solutions prior to column transport experiments. Results indicate that FG TiO_2 aggregate size (200-400 nm) remained consistent across solutions. Additionally, elution of FG and IG TiO_2 was greatest in septic effluent at the higher nanoparticle concentration (100 ppm). FG TiO_2 was well retained at the low (2 ppm) concentration in septic effluent suggesting that particles that escape the septic system may still be retained in drainage field before reaching the groundwater system, although eluted particles are highly stabilized. Findings provide valuable insight into the significance of the solution

environment at mediating differences observed between uniquely, engineered nanomaterials.

4.1 Introduction

Engineered nanomaterials (ENMs) represent an expanding class of nanosized materials that are used in numerous applications for matrix stabilization and enhancement of optical properties (Bishoge *et al.* 2018), including consumer products such as, foods (Abbas *et al.* 2009; Bouwmeester *et al.* 2009; Weir *et al.* 2012), cosmetics, coatings, pharmaceuticals, pigments, paints, and personal care products (Weir *et al.* 2012; Keller *et al.* 2013). Interestingly, multiple studies of the life cycle of consumer product based ENMs have reported that nanomaterials can be released from products during the lifespan of their usage with many reaching full scale wastewater treatment plants (WWTPs) (Benn *et al.* 2008; Keller *et al.* 2013). Titanium dioxide (TiO_2) ENMs, for example, are estimated to account for a large fraction (40-50%) (Keller *et al.* 2013) of engineered nanomaterials reaching WWTPs, due to frequent usage and release from foods and consumer products (Abbas *et al.* 2009; Bouwmeester *et al.* 2009; Gottschalk *et al.* 2009; Brar *et al.* 2010; Keller *et al.* 2013; Addo Ntim *et al.* 2018).

Full scale WWTPs reduce the loading of organics, nutrients, and other potential environmental contaminates before wastewater is released into the environment (Crittenden *et al.* 2012). Two to four primary treatment stages are typically incorporated at centralized facilities: preliminary treatment to remove large objects damaging to the plant, primary treatment using physical removal processes (i.e. sedimentation), secondary treatment uses biological or chemical processes for nutrient removal, and tertiary treatment further removes organics, pathogens, and parasites (Bitton 2005). Nanomaterials tend to aggregate and associate with biosolids within full scale WWTPs prior to sedimentation from treated effluent (Westerhoff *et al.* 2011; Westerhoff *et al.* 2013). However, in certain cases, though colloidal sized TiO_2 particles are well removed, up to 70% of nano TiO_2 was eluted from the full scale WWTP (Kiser *et al.* 2009). Additionally, TiO_2 nanoparticles released from WWTPs can remain bioavailable, which is evidenced by accumulation of small quantities (< 5µg/L) in fish just downriver of the treatment facility (Shi *et al.* 2016). Notably, TiO_2 ENMs detected in receiving waters were determined to be engineered and non-naturally occurring (Shi *et al.* 2016). These studies indicate that nanosized engineered TiO_2 can remain an environmental concern after leaving full scale WWTPs.

Septic wastewater treatment systems, alternatively, are a simpler microbially driven, anaerobic digester utilizing only the sedimentation and filtration aspects of larger treatment operations (Canter 1985). Sedimentation of suspended solids and microbial degradation of organics occurs in the septic tank and effluent slow trickle filtration occurs in the drainage field. Although septic systems are EPA regulated and effective when installed and maintained properly, they are often less rigorous than full scale WWTPs (Bitton 2005) and shortcomings in design and maintenance lead to system failure (EPA 1999). Notably, TiO_2 and other ENMs well studied and characterized in full scale WWTPs have largely been neglected in the onsite, septic systems (Taylor *et al.* 2015).

TiO_2 ENMs in poorly maintained septic systems, as opposed to WWTPs, suggests the possibility of greater elution from the onsite system (Waller *et al.* 2018) and shifts the focus to nanoparticle behavior in the drainage field after septic treatment.

Nanoparticle fate and transport in porous media is dependent on both solution chemistry and inherent particle characteristics (Gregory 2004; Chowdhury *et al.* 2011). TiO_2 nanoparticles characterized under idealized conditions indicated that food grade (FG) TiO_2, which is more commonly used in foods and consumer products (Weir *et al.* 2012), possessed a different isoelectric (IEP) point than a more commonly studied form of TiO_2 (FG: pH 3.5; industrial grade: pH 6) (Yang *et al.* 2014). Wastewater systems typically remain between pH 6-7 indicating that while industrial grade TiO_2 particles should be destabilized, FG TiO_2 should be stable with a negative charge based on IEP. Further, FG TiO_2 nanoparticles exhibit interesting stability across multiple ideal and complex solution conditions (Waller *et al.* 2017) potentially increasing its chances of remaining suspended in effluent eluted from the drainage field into the groundwater system. Essentially, transport mechanisms governing FG TiO_2 nanoparticle behavior in porous media after septic treatment are largely unknown and represent an important consideration regarding the environmental fate and transport of FG TiO_2.

This study aimed to characterize the transport behavior and mechanisms of FG TiO_2 particles in porous media indicative of conditions in post septic waste treatment. Two forms of TiO_2 nanoparticles were utilized to assess correlation between FG and a more commonly studied form of industrial grade (IG) TiO_2. Characterization of nanoparticles included particle size and mobility within each solution condition. Nanoparticle transport experiments were conducted using a cylindrical glass column wet packed with quartz sand grains. Breakthrough curves were then created to elucidate the influence of solution chemistry and particle concentration in FG TiO_2 porous media transport.

4.2 Experimental Protocols

4.2.1 Nanoparticle selection and characterization

Food grade (FG) and industrial grade (IG) TiO_2 were used, independently, for all experimental conditions of this study. Nanomaterial selection was largely determined by FG being a commonly found particle in domestic sewage (Weir *et al.* 2012; Keller *et al.* 2013), while the IG was used in this study as a control, as a most commonly studied TiO_2 particle (Yang *et al.* 2014). Interestingly, both grades have pathways to reach wastewater treatment facilities (Weir *et al.* 2012). Food grade TiO_2 are commercially available particles provided via Arizona State University, and have a primary particle size of 122 ± 48 nm and mostly anatase crystal structure (> 95%), additionally the particle carries an inorganic phosphate coating (Yang *et al.* 2014). Industrial grade TiO_2 are nanoparticles sourced from Sigma Aldrich (Aeroxide TiO_2 P25; Evonik Degussa Corporation, Essen, DE) and possess a primary particle size of 21 nm and a compound crystal structure of 75% anatase and 25% rutile (Yang *et al.* 2014; Waller *et al.* 2017). The isoelectric point of FG TiO_2 is within the range of pH 3.5 to 4, while IG TiO_2 is pH 6 in 10 mM monovalent electrolyte solutions (Yang *et al.* 2014; Waller *et al.* 2017).

Nanoparticles were prepared following previously described methods (Chowdhury *et al.* 2010). Briefly, a 200 ppm stock solution of TiO_2 nanoparticles was prepared and sonicated for 30 minutes prior to beginning experiments; then another 30 seconds just before each characterization experiment. Nanoparticle concentrations of 2 and 100 ppm were selected to approximate environmentally relevant levels and nanoparticle concentrations used in prior studies (Chowdhury *et al.* 2011). Although 0.2 ppm represents the high of TiO_2 in this environment (Qiu *et al.* 2016; Shi *et al.* 2016), 2 ppm was selected due to optical resolution limitations of the spectrophotometer.

Nanoparticle characterization included both hydrodynamic diameter and zeta-potential (ZP) measurements across each solution condition and TiO_2 concentration. Hydrodynamic diameter was determined using dynamic light scattering (ZetaPALS, Holtsville, NY, USA) at a wavelength of 661 nm and scattering angle of 90°. ZP was calculated using the Smoluchowski equation (Gregory 2004) converted using electrophoretic mobility measurements (ZetaPALS, Holtsville, NY, USA).

4.2.2 Solution chemistry and characterization

Treated wastewater released from septic treatment systems must first trickle through a drainage field of porous medium before entering the groundwater system (Canter 1985). Therefore, septic system wastewater effluent was the primary solution utilized in this study to replicate conditions present within the drainage field. Septic effluent was produced using a bench scale model septic tank and human colon that provided human fecal matter

(Marcus *et al.* 2013; Taylor *et al.* 2015; Waller *et al.* 2017). Conductivity and pH of the effluent were 570 μS/cm and 7.6, respectively and contained a microbial concentration of $5.01 \times 10^8 \pm 6.25 \times 10^7$. Additionally, 4 mM KCl with pH adjusted to 7.6 and 10 mM KCl (no adjustment, pH 6) were two monovalent electrolyte suspensions used to indicate the significance of organic matter and increased ionic strength, respectively. Conductivity is the measure of charge transferring capacity of a solution and an estimate of IS. Calibration curves were created to determine the molar concentration of KCl equaling septic effluent conductivity with the result being 4 mM. Food and industrial grade TiO_2 nanoparticles were introduced to septic effluent, 4 mM KCl (pH 7.6), and 10 mM KCl (pH 6) prior to being introduced into the sand column for transport experiments. Solution chemistries provided a comparison of the role played by organic material remaining in septic system (4 mM KCl, pH 7.6) and for the consideration of FG TiO_2 filtration behavior in relation to commonly studied literature conditions (10 mM KCl).

4.2.3 Column transport experiments

Column transport experiments were conducted to elucidate the porous media behavior of FG TiO_2. A borosilicate glass cylinder of dimensions 1.5 cm inner diameter and 5 cm length was wet packed with 275 um ultrapure quartz sand resulting in 0.46 porosity to simulate filtration through a septic drain field (Canter 1985). Column operation and setup are presented in greater detail elsewhere (Chowdhury *et al.* 2011). Flowrate was maintained at 2 ml/min which is commonly found in trickle flows and slow sand grain filtration (Crittenden *et al.* 2012). Greater than 10 pore volumes (PV) of Millipore deionized water (18.2 MΩ at 25 °C) were flushed through the column after sand was wet packed. This initial flush was followed by greater than 10 PV of the electrolyte solution (without nanoparticles) to fully saturate the porous media in the background solution. Six (6) PV of TiO_2 nanoparticle suspensions was then introduced followed by another 6 PV of the background electrolyte solution. Effluent was obtained from the column using 15 mL centrifuge tubes and fraction collector (CF 1 Fraction Collector, Spectrum Chromatography, Houston, TX). The nanoparticle suspension was constantly sonicated and stirred during TiO_2 introduction.

4.2.4 DLVO Theory

Derwin-Landau-Verwey-Overbeek (DLVO) theory was applied to provide insight on deposition mechanisms of both FG and IG TiO_2 in this porous media system. DLVO allowed for the determination of the significance of electrostatic repulsion and van der Waals interactions on the TiO_2 nanoparticles and quartz sand collector comprising the transport column. Sphere plate geometry was assumed for this TiO_2 quartz system due to the comparatively smaller diameters of nanoparticles compared with a quartz sand collector grain size of ~250-300 µm. DLVO theory was applied using the following equations for electrostatic repulsion (V_{edl}) and van der Waals forces (V_{vdw}) (2):

$$V_{edl} = \pi \epsilon_o \epsilon a_p \left\{ 2\psi_p \psi_c \ln \left[\frac{1 + exp\,(-kh)}{1 - exp(-kh)} \right] + ({\psi_c}^2 + {\psi_p}^2) \ln[1 - exp\,(-2kh)] \right\} \tag{1}$$

$$V_{vdw} = \frac{A_{102} a_p}{6h} \left(1 + \frac{14h}{\lambda} \right)^{-1} \tag{2}$$

where the following assumed parameters were utilized from previously published and measurements from this study: permittivity constant of free space (ε_o) was 8.854 x10^{-12} C/V/m, the dielectric constant of water (ε) was 78.5, surface potentials (ψ) determined by EPM measurements transformed to zeta-potentials using the Smoluchowski equation for the particle (p) and collector (c) (Elimelech *et al.* 1995). Inverse Debye length and separation distance are denoted by (κ) and (h), respectively (Gregory 2004). The Hamaker constant (A_{102}) selected for a quartz TiO_2 system was 10^{-20} (Fatisson *et al.* 2009) and particle radius (a_p) was populated by hydrodynamic diameter values (Figure 4.1). Lastly, characteristic wavelength (λ) was assumed to be 100 nm (Elimelech *et al.* 1995).

4.2.5 Statistical Analysis

Student t-test was used to determine statistical significance for nanoparticle characterization and breakthrough curves. An alpha value of 0.05 ($p\text{-value} < 0.05$) indicated statistically significant differences resulted in the FG or IG TiO_2 nanoparticle suspensions.

4.3. Results

4.3.1 Nanoparticle Characterization

The hydrodynamic diameter of each type of TiO_2 particle are presented in Figure 4.1. Notably, IG and FG TiO_2 vary little in their respective mean diameters between both KCl suspensions. Industrial grade TiO_2 developed a significantly larger ($p < 0.05$) hydrodynamic diameter than FG TiO_2 in both 4 mM and 10 mM KCl, which is similar to previous research that found IG to form larger aggregates than FG (Waller *et al.* 2017).

Contrary size relationships by particle concentration were observed in KCl between FG and IG TiO_2 hydrodynamic diameters with 100 ppm IG TiO_2 developing significantly larger aggregates than 2 ppm (> 1200 nm to < 600 nm, respectively) while 100 ppm FG formed significantly smaller aggregates than 2 ppm (< 282.5 ± 7.5 nm to > 358.3 ± 3.2 nm). Suspending the particles in septic effluent resulted in the development of very similar sizes between IG and FG TiO_2. Septic effluent appears to stabilize IG TiO_2 particle sizes compared to both KCl solutions, while the size FG TiO_2 was determined to be independent of solution chemistry.

The electrophoretic mobility for both grades of TiO_2 particles suspended in the three solution chemistries are presented in Figure 4.2. The EPM of FG TiO_2 was negative in all 3 solution conditions and the value was independent of particle concentration. The greatest magnitude of EPM for FG was the 4 mM KCl solution, followed by the 10 mM suspension, while the FG suspended in the septic effluent had the lowest EPM. The IG particle EPM trends were similar across the two KCl suspensions. The low concentration IG particles had negative EPM values, while the higher concentrations had positive EPM values, yet irrespective of concentration the IG particles developed equal, negative, and lower magnitude EPM values when suspended in septic effluent (Figure 4.2). Indeed, both grades of TiO_2 at both concentrations exhibited no differences in EPM when suspended in septic effluent. Septic effluent may exhibit a capacity for EDL compression even at low IS (570 μS/cm, ~4 mM) as both TiO_2 types and concentrations exhibit reduced EPM from either KCl solution (Zhang *et al.* 2008).

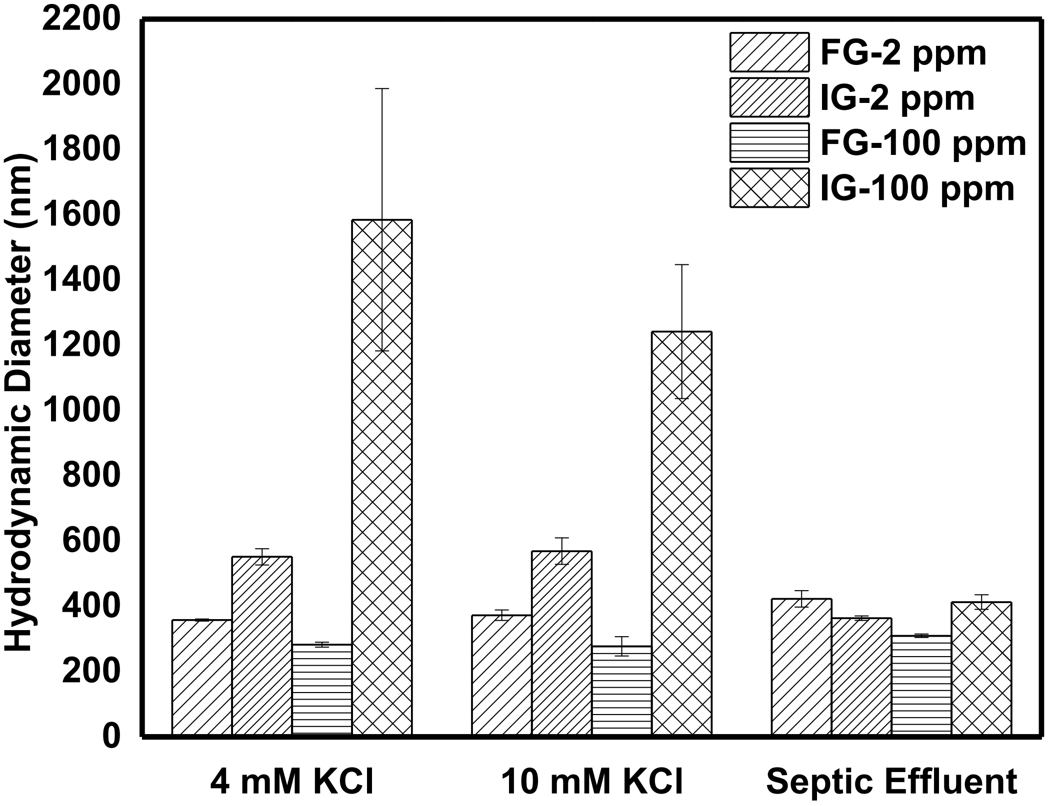

Figure 4.1 Hydrodynamic diameters for food grade (FG) and industrial (IG) titanium dioxide particles in 4mM KCl at pH 7.6, 10 mM KCl pH 6, and septic effluent pH 7.6. Errors bars represent standard deviation of triplicate measurements.

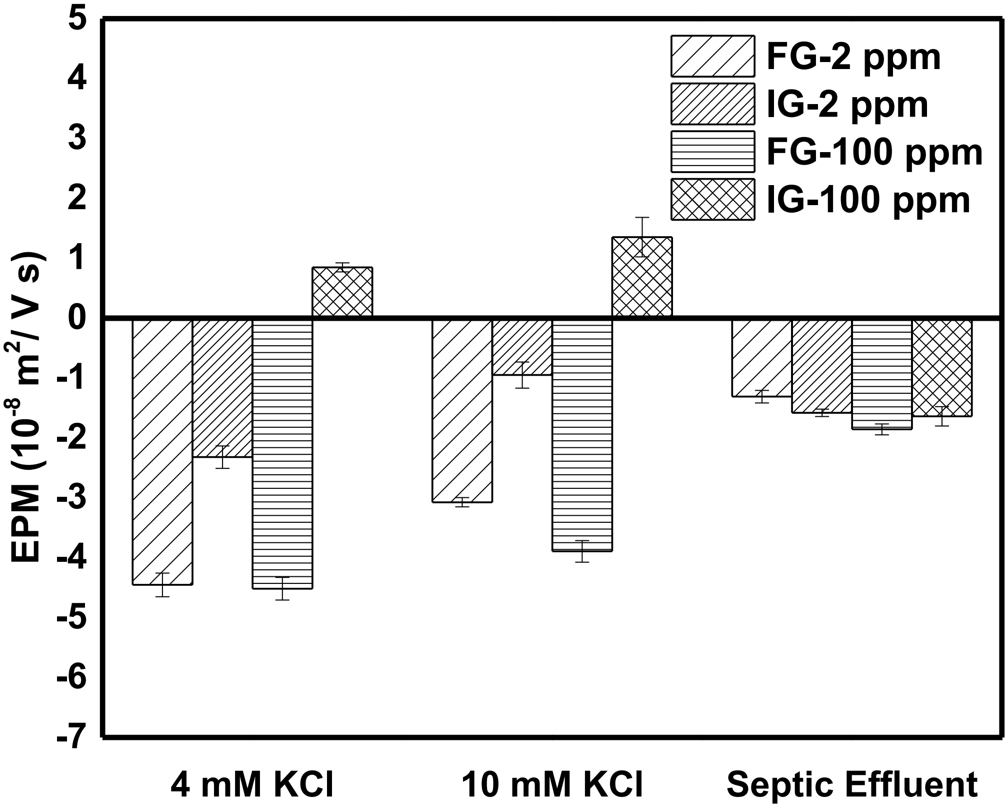

Figure 4.2 Electrophoretic mobility (EPM) for food grade (FG) and industrial (IG) titanium dioxide particles in 4mM KCl at pH 7.6, 10 mM KCl pH 6, and septic effluent pH 7.6. Errors bars represent standard deviation of triplicate measurements.

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4.3.2 Column Transport Experiments

Breakthrough curves for food and industrial grade TiO_2 in each solution condition are provided in Figure 4.3. Results indicate that FG TiO_2 was eluted more than IG in 4mM KCl although in 10 mM KCl both nanoparticle types were well retained (Figure 4.3a and 4.3b). Nanoparticle concentration had little effect on IG retention in 10 mM KCl except in the case of 100 ppm IG TiO_2 where a small increase in particles eluted was observed (0.1 ± 0.0). Conversely, FG TiO_2 was far more impacted by particle concentration where FG 100 ppm (max: 0.4 ± 0.0) was much greater than FG 2 ppm (max: 0.0 ± 0.0) when suspended in 4 mM KCl. In septic effluent, differences in nanoparticle behavior were observed for both FG and IG TiO_2 when compared to the monovalent solutions (Figure 4.3c). Septic effluent resulted in high elution values for all nanoparticles and concentrations except for FG 2 ppm. Notably, IG TiO_2 displayed an increased tendency for elution than those observed in 4 and 10 mM KCl. TiO_2 particle concentration played a major role under septic effluent conditions as both IG and FG TiO_2 exhibited similar behavior at the 100 ppm TiO_2 concentrations and poor retention was observed at 100 ppm (max: 0.8 ± 0.1) compared to 2 ppm (max: 0.1 ± 0.0). Results indicate that septic effluent conditions facilitate increased elution of FG and IG TiO_2 nanoparticles compared to the monovalent electrolyte suspensions, though high retention is observed at environmentally relevant concentrations (< 2ppm).

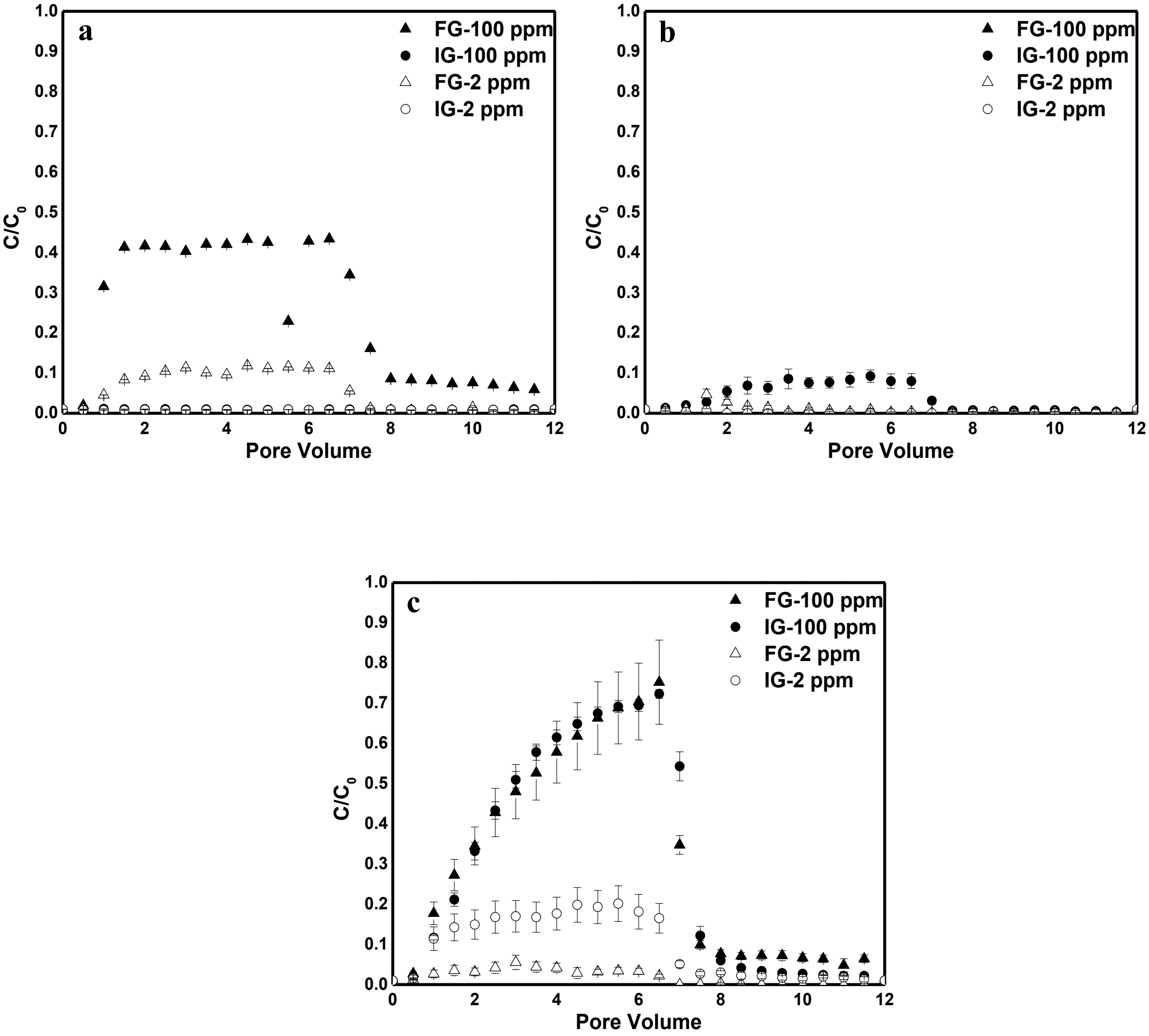

Figure 4.3 Breakthrough curves for food (FG) and industrial grade (IG) TiO_2 nanoparticles introduced to (a) 4mM KCl, pH 7.6, (b) 10 mM KCl, pH 6, and (c) Septic effluent. C_0 and C represent influent and effluent concentrations of TiO_2. Error bars represent standard error of triplicate measurements.

4.3.3 DLVO calculations

Total interaction energy profiles for food and industrial grade TiO_2 developed for 4 mM and 10 mM KCl are presented in Figure 4.4 as a function of nanoparticle concentration and separation distance. Unfavorable conditions (> 435 kT) for deposition were observed for both FG and IG TiO_2 nanoparticles in 4 mM KCl apart from IG 100 ppm, where the deep primary minimum demonstrates that very favorable conditions developed (Figure 4.4a: -5292kT). For the 10 mM KCl suspensions, TiO_2 nanoparticle type was significant with FG TiO_2 remaining in unfavorable deposition conditions, although reduced from observations in 4 mM, while IG 2 ppm became much closer to favorable deposition (Figure 4.4b). Additionally, nanoparticle concentration affected total interaction energy with IG TiO_2 more than FG TiO_2. FG TiO_2 remained unfavorable in 4 mM KCl at both particle concentrations and possessed similar interaction energy (FG 2 ppm: 225 kT; FG-100 ppm: 195 kT). Conversely, nanoparticle concentration was the difference between favorable and unfavorable deposition conditions for IG TiO_2 in 4mM and 10 mM KCL, with unfavorable conditions for IG 2 ppm, while IG-100 was favorable. Results of interaction profiles suggests that more favorable conditions for FG and IG TiO_2 nanoparticle deposition, at both 2 and 100 ppm concentrations, would exist in 10 mM KCl than 4 mM KCl.

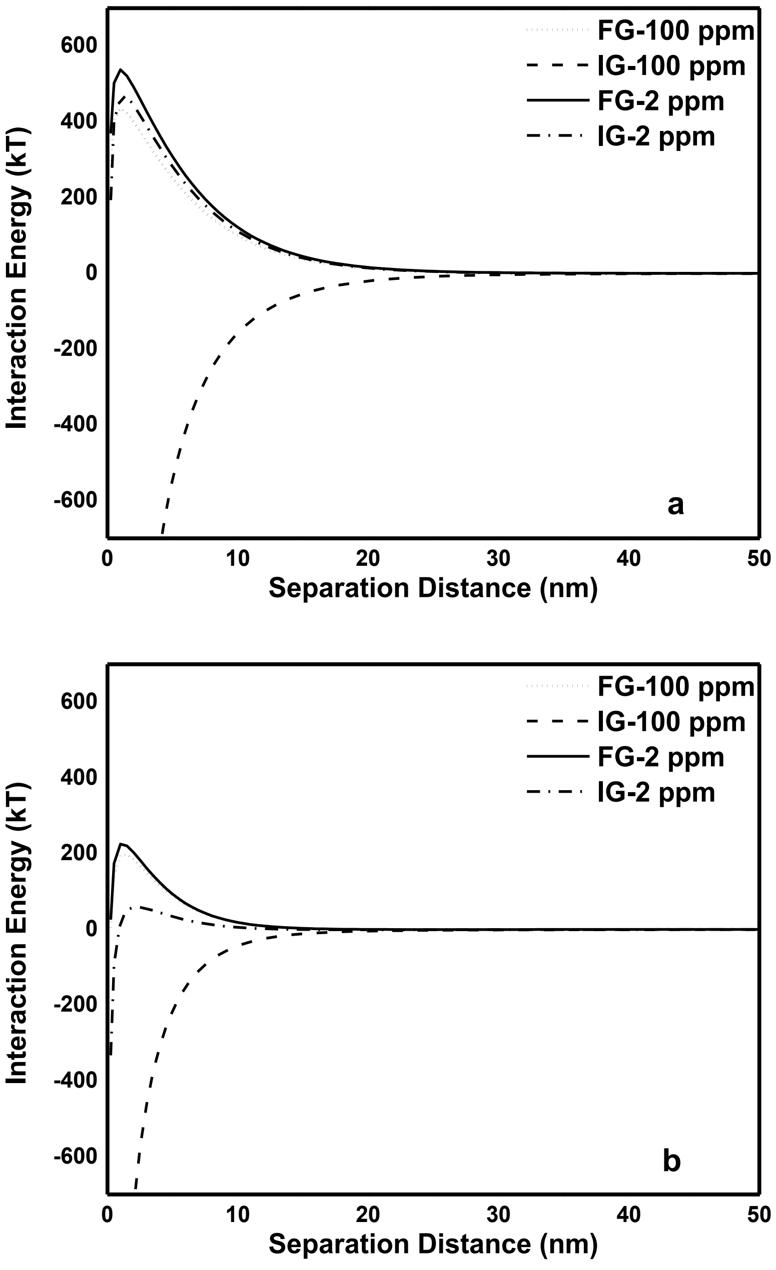

Figure 4.4 Total interaction energy profiles generated by Derwin-Landau-Verwey-Overbeek (DLVO) theory for food (FG) and industrial grade (IG) TiO_2 nanoparticles and the quartz sand collector surface in both (a) 4mM KCl, pH 7.6 and (b) 10 mM KCl, pH 6, as a function of separation distance

4.4. Discussion

4.4.1 Nanoparticle Characterization

Engineered nanomaterial interactions, and environmental behavior as a result, are very much dependent on nanoparticle stability and aggregation (Chen *et al.* 2007; Chen *et al.* 2007). High electrophoretic mobility (-4.5 ± 0.2 10^{-8} m^2/V s) of FG TiO_2 nanoparticles introduced to weak, monovalent electrolyte conditions is suggestive of a very stabilized nanoparticle (Figure 4.2). Similar mobility displayed by FG TiO_2 was also observed in concentrated colon medium comprising proteins, carbohydrates, and mono- and divalent salts, at even at high IS (~200 mM) (Waller *et al.* 2017). . Between 4 and 10 mM KCl, the hydrodynamic diameter of FG TiO_2 remains in the range of 270 to 360 nm while IG-TiO_2 increases to well over 1000 nm in both solutions at IG 100 ppm, which also aligns with findings from previous work conducted in high IS, multivalent electrolyte solutions (Waller *et al.* 2017). FG TiO_2 hydrodynamic diameters in 4 mM and 10 mM KCl of support EPM observations of a stabilized aggregate showing that in, FG TiO_2 maintains smaller aggregate sizes than IG TiO_2 (Figure 4.1). Di- and multivalent electrolyte suspensions are well understood to compress the electrical double layer surrounding a nanoparticle (French *et al.* 2009) and while this was observed previously with IG TiO_2, FG TiO_2 continues to respond differently in idealized and complex solutions highlighting the significance of inherent differences (Waller *et al.* 2017).

Suspension pH is an additional factor capable of influencing nanoparticle stability, where particles near the isoelectric points (IEPs) are destabilized and more readily aggregate (Gregory 2004; Chowdhury *et al.* 2011). The IEPs of FG and IG TiO_2 have been reported as pH 3.5 and 6, respectively (Yang *et al.* 2014). Based on IEPs and nanoparticle concentration, the largest TiO_2 aggregates would be expected to form in IG-100 ppm, although this was not the case (Figure 4.1). Slightly larger IG-100 ppm aggregates observed in 4 mM KCl at pH 7.6 may potentially result from a positive, and reduced stability compared to 10 mM, resulting in conditions that facilitate nanoparticle aggregation (Figure 4.2) (Zhang *et al.* 2008; Keller *et al.* 2010).

In wastewater treatment, organic coatings are expected to develop on nanoparticles surfaces and can impart a stabilizing effect that minimizes further aggregation (Chen *et al.* 2007; Zhang *et al.* 2008; Chowdhury *et al.* 2013; Chowdhury *et al.* 2013). Contrary to the monovalent solutions, septic effluent represented an environment that reduced the mobility of both TiO_2 types; however, whereas the hydrodynamic diameter of IG TiO_2 nanoparticles decreased, FG TiO_2 aggregates became slightly larger at both 2 and 100 ppm nanoparticle concentrations (Figures 4.1 and 4.2, respectively). Reductions in EPM and hydrodynamic diameter have been attributed to steric hindrance occurring from organic matter bound to the nanoparticle surface (Chen *et al.* 2007; Chowdhury *et al.* 2012) which can explain the observations for IG TiO_2. These findings suggest that FG TiO_2 would have low retention in porous media filtration increasing the possibility of reaching groundwater.

4.4.2 Column Transport Experiments

Findings from column breakthrough curves reveal an expectedly high rate of elution of FG TiO_2 nanoparticles in septic system effluent at high concentrations (Figure 4.3c) (Kiser *et* *al.* 2009; Westerhoff *et al.* 2011; Giese *et al.* 2018). However, environmentally relevant concentrations of nanoparticles released from septic tank may not pose an immediate risk to groundwater as FG 2 ppm was well retained inside the column. IG TiO_2 conversely exhibited a high degree of elution at both 2 and 100 ppm concentrations compared to the monovalent electrolyte solutions. Poor retention of IG TiO_2 in septic effluent compared to 4 mM and 10 mM KCl may potentially result from steric hindrance minimizing aggregation and deposition allowing particles to remain in suspension (Chen *et al.* 2007; Zhang *et al.* 2008; Chowdhury *et al.* 2012; Chowdhury *et al.* 2013). Interestingly, findings of the current study suggest that high stability of TiO_2 ENMs may be a major factor in the frequency of their detection in WWTPs compared to other nanomaterials (Westerhoff *et al.* 2013).

4.4.3 DLVO experiments

Derwin-Landau-Verwey-Overbeek (DLVO) theory was used to provide insight on the influence of van der Waals and electrostatic repulsive forces on TiO_2 nanoparticle interactions with quartz sand collectors during porous media transport (Figure 4.4) (Bradford *et al.* 2009; Chowdhury *et al.* 2011). Total interaction energy profiles determine whether favorable conditions exist for TiO_2 nanomaterials to deposit onto collector grains. Septic effluent exceeds the idealized solution assumption used for DLVO theory (Elimelech *et al.* 1995), so 4 mM KCl (pH 7.6) was used instead to consider effects of IS and nanoparticle concentration on interaction energy between FG TiO_2 and the quartz collector as a function of distance.

FG TiO_2 has unfavorable conditions for deposition at 2 and 100 ppm in both solutions with higher interaction energy in 4 mM KCl (max: 537 kT, FG 2 ppm) (Figure 4.4). Considering FG TiO_2 has an IEP of 3.5, exposure to pH 7.6 would result in both negatively charged nanoparticles and the collector surface ultimately inhibiting deposition (Elimelech *et al.* 1995; Gregory 2004). Similarly, FG 2 ppm TiO_2 also possesses the highest interaction energy (max: 225 kT) although reduced from 4 mM KCl. Although, FG 2 ppm developed the most unfavorable conditions for deposition in both solutions based on DLVO theory, it was observed to be the most retained nanoparticle filtration column when suspended in the septic effluent (Figure 4.4). Additionally, IG 100 ppm would be expected to have highly favorable deposition in 10 mM KCl and 4 mM KCl as shown in Figure 4.4, yet high elution was recorded in septic effluent (Figure 4.3). This suggests that organic material in water sources can potentially impart environmentally specific characteristics (Chen *et al.* 2007; Chowdhury *et al.* 2013) that may take precedent over inherent nanoparticle characteristics in governing nanoparticle behavior.

Engineered nanomaterials are an expanding class of emerging contaminants to environmental systems including water bodies and water treatment facilities. TiO_2 nanoparticles commonly incorporated into food and consumer products and subsequently released during usage have been detected at wastewater treatment facilities. As such, the impact of TiO_2 nanomaterials in wastewater treatment facilities was assessed using the environmentally relevant form of TiO_2 (food grade) and concentration (< 2 ppm) to elucidate filtration behavior of food grade TiO_2 nanoparticles after exiting the septic treatment system. Notably, septic effluent resulted in increased elution of both industrial and food grade TiO_2 at higher nanoparticle concentrations. FG TiO_2 was well retained at 2 ppm concentration in septic effluent indicating that particles that escape the septic system may still be removed from the waste stream before reaching groundwater. However, particles that are eluted from the drainage field into the groundwater system are likely to be very stabilized in suspension likely resulting in greater transport. Findings of the current study provide essential insight into the significance of the solution conditions that nanoparticles are introduced to in that the potential exists for the environment to mediate differences observed between uniquely, engineered nanomaterials. Future research into filtration of FG TiO_2 will elucidate governing mechanisms that facilitate and suppress differing behaviors between nanomaterials to better understand mechanisms governing transport of nanoparticles in porous media.

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Chapter 5

Summary and Conclusions

Chapter 5: Summary and Conclusions

The primary objective of this doctoral research was to determine whether differences observed in idealized systems in nanomaterial behavior, imparted by their engineered nature, manifest in more complex systems and solutions. Specifically addressed was the environmental significance of food grade TiO_2 nanoparticles within environmental systems that are microbially driven, as well as, differences in environmental responses of those microbial systems between different forms of TiO_2 nanomaterials. This objective was fulfilled by characterizing the impact of the TiO_2 particles in three environmental systems with known microbial presence: the human large intestine, the septic wastewater treatment system, and drainage field post septic treatment. Nanoparticle leaching from food and consumer products during usage and the prevalence of TiO_2 nanoparticles at wastewater treatment facilities led to the selection of the more environmentally prevalent food grade TiO_2 and the more commonly investigated industrial grade TiO_2 for comparison of functional differences. Phenotypic, genotypic, and biochemical parameters were used to monitor deviations in microbial community function.

Chapter 2 introduced food and industrial grade TiO_2 nanomaterials into a model human colon bioreactor to characterize phenotypic, biochemical, and compositional parameters of the microbial community. Findings revealed an inhibition in the presence of both particles of a natural shift in microbial composition from Proteobacteria to Firmicutes occurring during control conditions due to diet, with food grade TiO_2 introduction having the greater effect. Further, lower colonic pH (< 5) as compared to the control (> 5) developed in both

TiO_2 exposures resulted in, with food grade exposures recording the largest reduction (~ pH 4). TiO_2 nanomaterials may have little direct effect on microbial stability based on similar trends in microbial community hydrophobicity and electrophoretic mobility between baseline, food, and industrial grade exposures. Results of this study indicate that inherent physical and chemical properties of the two TiO_2 forms may indeed produce different microbial responses, which is significant when considering environmental exposure and risk, as well as the design of environmental fate and toxicity studies.

Chapter 3 considers phenotypic and compositional responses of anaerobic wastewater bacteria to food and industrial grade TiO_2 introduction to the septic treatment system, as well as, septic system function. Notably, FG and IG TiO_2 exposure resulted in distinct responses in septic effluent quality, microbial composition, and relative abundance. Exposure to IG TiO_2 may prove beneficial to the septic system in the short term by facilitating VFA production. Conversely, FG TiO_2 appears more inhibitory to anaerobic digestion by interference in organic matter mineralization. Far greater diversity was observed in the relative abundance of genera comprising the microbial community during FG TiO_2 exposures than IG TiO_2, suggesting that FG TiO_2 may alter microbial relationships affecting anaerobic digestion efficiency. Exposure of the septic system to IG TiO_2 particles had minimal effect on the composition of the denitrifying-dominant microbial community, while the FG exposures shifted the community structure to genera of mixed functionality, which is less effective at wastewater treatment. Lastly, inversely related tendencies in biofilm growth were the result of acute exposure of the septic microbial community to FG and IG TiO_2 particles. IG exposure lead to increased production of biofilm, while FG exposure lead to reduced biofilm production. Valuable insight that nano-FG TiO_2 may cause considerable changes to microbial function in septic systems is gained from these findings and that understanding downstream effects requires studying ecologically relevant forms of nanoparticles.

Chapter 4 explored removal mechanisms and behavior of food grade TiO_2 during filtration through porous media after septic system treatment. FG TiO_2 aggregate size (200-400 nm) remained consistent across each solution condition (4mM KCl, 10 mM KCl, and septic effluent). Elution of FG and IG TiO_2 was the greatest in septic effluent at the higher nanoparticle concentration (100 ppm). Column breakthrough curves further indicate that FG TiO_2 nanoparticles that escape the septic tank may still be prevented from reaching groundwater via retention in the drainage field based on minimal elution at the low (2 ppm) nanoparticle concentration in septic effluent. However, eluted particles are highly stabilized and potentially remain bioavailable. Findings provide valuable insight into the significance of the solution environment at mediating differences observed between uniquely, engineered nanomaterials.

Findings of this doctoral study provide key insight regarding how the engineered nature of a nanoparticle can impart unique characteristics that result in distinctly different responses from microbially driven environmental systems. Notably, food grade TiO_2 possessed a remarkable stability within the solution conditions of each environmental system, whereas industrial grade TiO_2 was far more susceptible to aggregation in the selected range of solution chemistries. Although the particles have the same molecular formula, their environmental impact suggests they should be classified separately. Next, the presence of TiO_2 particles, much more so in the case of food grade, introduced to the modeled human gut microbial community prevented a natural shift in the microbial composition occurring due to diet. This indicates that food grade TiO_2 demonstrated a greater ability to alter the nature of this environmental system. Lastly, industrial grade TiO_2 exhibited an ability to facilitate the organic degradation occurring in the septic system resulting in a higher quality effluent. This suggests industrial grade TiO_2 nanoparticles may have other beneficial qualities than photocatalysis for wastewater treatment. This dissertation demonstrates the significance of TiO_2 nanomaterials in microbial environments and provides information to better prepare for an expanding class of uniquely behaving nanoparticles. Further work would be valuable to study real time, characterization and visualization methods of organic coatings attached to nanoparticle surfaces to note unique interactions with media between different particle types.